A STUDY ON CRYPTOCOCCAL MENINGITIS IN PATIENTS WITH ACQUIRED IMMUNODEFICIENCY SYNDROME

Dissertation submitted to

THE TAMILNADU DR.M.G.R.MEDICAL UNIVERSITY

in partial fulfillment of the regulations

for the award of the degree of

M.D. (MICROBIOLOGY)

BRANCH – IV

MADRAS MEDICAL COLLEGE

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI – TAMILNADU

APRIL 2013

CERTIFICATE

This is to certify that this dissertation titled **"A STUDY ON CRYPTOCOCCAL MENINGITIS IN PATIENTS WITH ACQUIRED IMMUNODEFICIENCY SYNDROME"** is a bonafide record of work done by **DR.J.DURDANA PARVEEN,** during the period of her post graduate study from May 2010 to April 2013 under guidance and supervision in the Institute of Microbiology, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai-600003, in partial fulfillment of the requirement for **M.D.MICROBIOLOGY** degree examination of The Tamilnadu Dr. M.G.R. Medical University to be held in April 2013.

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DECLARATION

I declare that the dissertation entitled **"A STUDY ON CRYPTOCOCCAL MENINGITIS IN PATIENTS WITH ACQUIRED IMMUNODEFICIENCY SYNDROME"** *submitted by me for the degree of M.D. is the record work carried out by me during the period of* ***October 2011 to September 2012*** *under the guidance of Prof.* ***Dr.S.THASNEEM BANU, M.D.,*** *Professor of Microbiology, Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to The Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfillment of the University regulations for the award of degree of M.D., Microbiology (Branch IV) examination to be held in April 2013.*

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ACKNOWLEDGEMENT

*I humbly submit this work to the **Almighty** who has given the health and ability to pass through all the difficulties in the compilation and proclamation of this blue print.*

*I wish to express my sincere thanks to our Dean, **Dr.V.KANAGASABAI M.D.,** for permitting me to use the resources of this institution for my study.*

*I owe special thanks to **Dr.G.JAYALAKSHMI M.D., D.T.C.D.,** Director & Professor, Institute of Microbiology for her constant encouragement, innovative ideas, and timely suggestions during my work.*

*I express my sincere thanks to **Dr.C.CHANDRASEKAR M.D., D.T.C.D.,** Superintendent and **Dr.O.R.KRISHNARAJASEKHAR M.D., D.T.C.D.,** Deputy Superintendent, Government Hospital of Thoracic Medicine for permitting me to use the resources of their institution for my study.*

*I feel indebted to **Dr.S.THASNEEM BANU M.D.,** Institute of Microbiology for her constant support, invaluable suggestions, and erudite guidance in my study and for being a source of inspiration in my endeavours.*

*I express my thanks and gratitude to our former Directors, Prof. **Dr.G.SUMATHI M.D., Ph.D.,** Prof. **Dr.R.MANJULA M.D.,** Prof. **Dr.M.MOHAMED MEERAN M.D., D.V.L.,** and former Director I/c **Dr.S.GEETHALAKSHMI M.D., Ph.D.,** for their guidance and support.*

*I would like to thank my Professors, **Dr.S.G.NIRANJANA DEVI M.D., D.G.O., Dr.S.VASANTHI M.D., Dr.T.SHEILA DORIS M.D.,** and **Dr.U.UMA DEVI M.D.,** for their valuable assistance in my study.*

I would also like to thank my former Professors, ***Dr.J.SASIKALA M.D.,*** *and* ***Dr.N.DEVASENA M.D.,*** *for their valuable guidance in my study.*

*I extend my whole hearted gratitude to my Assistant professor****, Dr.DAVID AGATHA M.D.,*** *for her valuable guidance and everlasting support in my study.*

I also express my sincere thanks to our Assistant professors, ***Dr.LATA SRIRAM M.Sc.,Ph.D., Dr.R.DEEPA M.D., Dr.N.RATHNA PRIYA M.D., Dr.K.G.VENKATESH M.D., Dr.C.S.SRIPRIYA M.D., Dr.N.LAKSHMIPRIYA M.D.,D.C.H., Dr. USHA KRISHNAN M.D.,*** *and* ***Dr.B.NATESAN M.D.,D.L.O.,*** *for their support in my study.*

I would like to thank our former Assistant Professors, ***Dr.EUPHARASIA LATHA, M.D.,D.G.O., Dr. P.BALAPRIYA ,M.D.,D.A., Dr.T.SABEETHA M.D., D.G.O.,*** *and* ***Dr.UMA PANDIYAN M.D.,*** *for their support during my study.*

I extend my sincere thanks to ***Dr.ARUNALOKE CHAKRABARTI M.D.,*** *Professor of Mycology Divison, PGIMER-Chandigarh for kindly providing the ATCC control strains for use in this study.*

I also sincerely thank ***Dr.JOY SAROJINI MICHAEL M.D.,*** *Professor I/c Mycology section, CMC Vellore for kindly providing C.gattii (serotype B) for use in this study.*

I express my thanks to ***Dr.J.SURIYA KUMAR M.D.,*** *for his valuable suggestions during my study.*

I would like to thank all my colleagues and all staff of Institute of Microbiology, Madras Medical College and Chennai-3 for their help and encouragement.

I also express my special thanks to all the laboratory staff of Government Hospital of Thoracic Medicine for their support in my study.

I would like to thank the Institutional Ethics Committee, Madras Medical College and Institutional Review Board, Government Hospital of Thoracic Medicine for approving my study.

I am indebted to all my family members who have been solid pillars of everlasting support and encouragement and for their heartfelt blessings.

Finally I am indebted to acknowledge the people who had enrolled in my study and gave their maximum co-operation and consent for the successful completion of the study.

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APRIL 2013

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Assignment title	Medical
Author	Durdana Parveen 20102104 M.D. Microbiology
E-mail	durdanaparveen@yahoo.com
Submission time	25-Dec-2012 02:08PM
Total words	17137

First 100 words of your submission

A STUDY ON CRYPTOCOCCAL MENINGITIS IN PATIENTS WITH ACQUIRED IMMUNODEFICIENCY SYNDROME Dissertation submitted to THE TAMILNADU DR.M.G.R.MEDICAL UNIVERSITY in partial fulfillment of the regulations for the award of the degree of M.D. (MICROBIOLOGY) BRANCH – IV MADRAS MEDICAL COLLEGE, THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY CHENNAI – TAMILNADU APRIL 2013 1 CERTIFICATE This is to certify that this dissertation titled “A STUDY ON CRYPTOCOCCAL MENINGITIS IN PATIENTS WITH ACQUIRED IMMUNODEFICIENCY SYNDROME” is a bonafide record of work done by DR.J.DURDANA PARVEEN, during the period of her post graduate study from May 2010 to April 2013 under guidance and supervision in the Institute of...

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Introduction

INTRODUCTION

Cryptococcus neoformans is an encapsulated heterobasidiomycetous fungus that has progressed from being a rare human pathogen to become a common worldwide opportunistic pathogen in immunocompromised hosts[75]. Although the name *Cryptococcus* literally means "hidden seed", the hitherto less known pathogen came into limelight with the advent of the AIDS pandemic. The genus *Cryptococcus* comprises of over 70 species but human infection is seldom caused by species other than *C.neoformans* and *C.gattii*[47]. Occasional cases of infections due to *C.laurentii* (Lynch *et al*, 1981)[59], *C.albidus* (Horowitz *et al*, 1993)[35] and *C.adeliensis* (Rimek *et al*, 2004)[83] have been reported but isolation of other cryptococcal species from clinical specimens requires both culture and histological proof of invasion before attributing disease to them. In general, *C.neoformans* is associated with infection in immunocompromised hosts while *C.gattii* is associated with infections in immunocompetent hosts[76].

The spectrum of human cryptococcosis varies broadly from asymptomatic colonization of the respiratory tract to widespread disseminated infection. Cryptococcal meningoencephalitis (CM) is the most severe, life threatening clinical presentation requiring immediate antifungal therapy in affected patients. CM is an AIDS defining condition in patients infected with HIV and generally occurs when the CD4 count falls below 100 cells /µl[76]. During the early 1990s, about 5-15% of patients with AIDS suffered from CM which accounted for almost 2/3rd of all culture proven cases of meningitis. Although the incidence of CM has declined in developed countries due to widespread use of HAART and prophylactic medications; CM still continues to be the leading cause of meningitis in HIV patients in developing countries. At present, the global burden of CM as estimated by CDC is 1 million new cases/ yr with about 1,33,600 cases occurring in South

east Asia[43]. According to recent WHO estimates, cryptococcosis accounts for 13-44% of deaths in HIV infected cohorts in resource limited countries[81]. Even with optimal treatment, the 10 week mortality rate of HIV associated CM is high ranging from 10-25% and reaches upto 65% in resource poor settings.[12, 39,74] In Sub-Saharan Africa alone, >5, 00,000 deaths occur each year due to *Cryptococcus* which may exceed those attributed to tuberculosis[82]. In India, the prevalence of meningitis due to *Cryptococcus* in HIV patients has shown a remarkable decline from 45-60% in the beginning of 21st century to 3-16% by the beginning of this decade[93].

C.gattii which has long been considered to infect only immunocompetent hosts has started to cause infections in AIDS patients adding to the burden of cryptococcal infection in this cohort .[14, 17] Since neurological complications are more with *C.gattii*, identification of this species needs to be prioritized. An additional concern is the emergence of drug resistance in *Cryptococcus* to antifungal agents in recent years[48]. Though the reports are sparse, routine testing for antifungal susceptibility testing of clinical isolates is necessary to obtain baseline data and observe any shift in sensitivity pattern.

Aims and objectives

AIMS AND OBJECTIVES

1. Rapid identification of *Cryptococcus* as the etiologic agent of meningitis in HIV positive patients using microscopic techniques and latex agglutination test

2. Isolation of *Cryptococcus* in culture from the CSF of the affected patients and also from other sites (in cases of disseminated infection)

3. Speciation of the cryptococcal isolates using phenotypic methods

4. Determination of susceptibility pattern of cryptococcal isolates to antifungal agents and comparison of MIC values obtained by E- test with those of microbroth dilution method

Review of literature

REVIEW OF LITERATURE

HISTORY

The first isolation of *Cryptococcus* from the environment was in 1894 by Sanfelice who recovered encapsulated yeast from peach juice and named it as *Saccharomyces neoformans*. In the subsequent year, Otto Busse & Buschke independently reported the isolation of yeast from a 31yr old woman in Germany with an ulcer over her tibia[77]. Buschke called the organism *Saccharomyces subcutaneous tumefaciens*. However Vuillemin transferred the yeast to the genus *Cryptococcus* in 1901 as he did not find ascospores characteristic of *Saccharomyces* in the isolate and named it as *C.hominis*. In 1905, Van Hansemann observed *Cryptococcus* in a case of meningitis and described the presence of yeast in gelatinous cysts in his post mortem report. Verse in 1914 recognised *Cryptococcus* antemortem in a case of leptomeningitis in a 29yr old woman. Cutler and Stoddard in 1916 delineated the clinical and pathologic differences between cryptococcosis, blastomycosis and other mycoses. They erroneously assumed that the capsule of the organisms were cysts in the tissue caused by digestive action of the fungus and named the organism *Torula histolytica* and the disease was known for a long time as Torulosis[77]. As most of the cryptococcal isolates were initially from Europe, the clinical entity of cryptococcosis was referred as European Blastomycosis too. Rhoda Benham in 1935 attempted to categorise 27 isolates of pathogenic cryptococci based on morphology, fermentation and serological studies[75]. She concluded that there was only one species and suggested retaining the name *Cryptococcus hominis*. In 1952 Lodder and Kreger Van Rij emphasised the priority of the name *C.neoformans* which finally became the accepted name. In 1976, Kwon-Chung discovered and characterized the sexual stage of

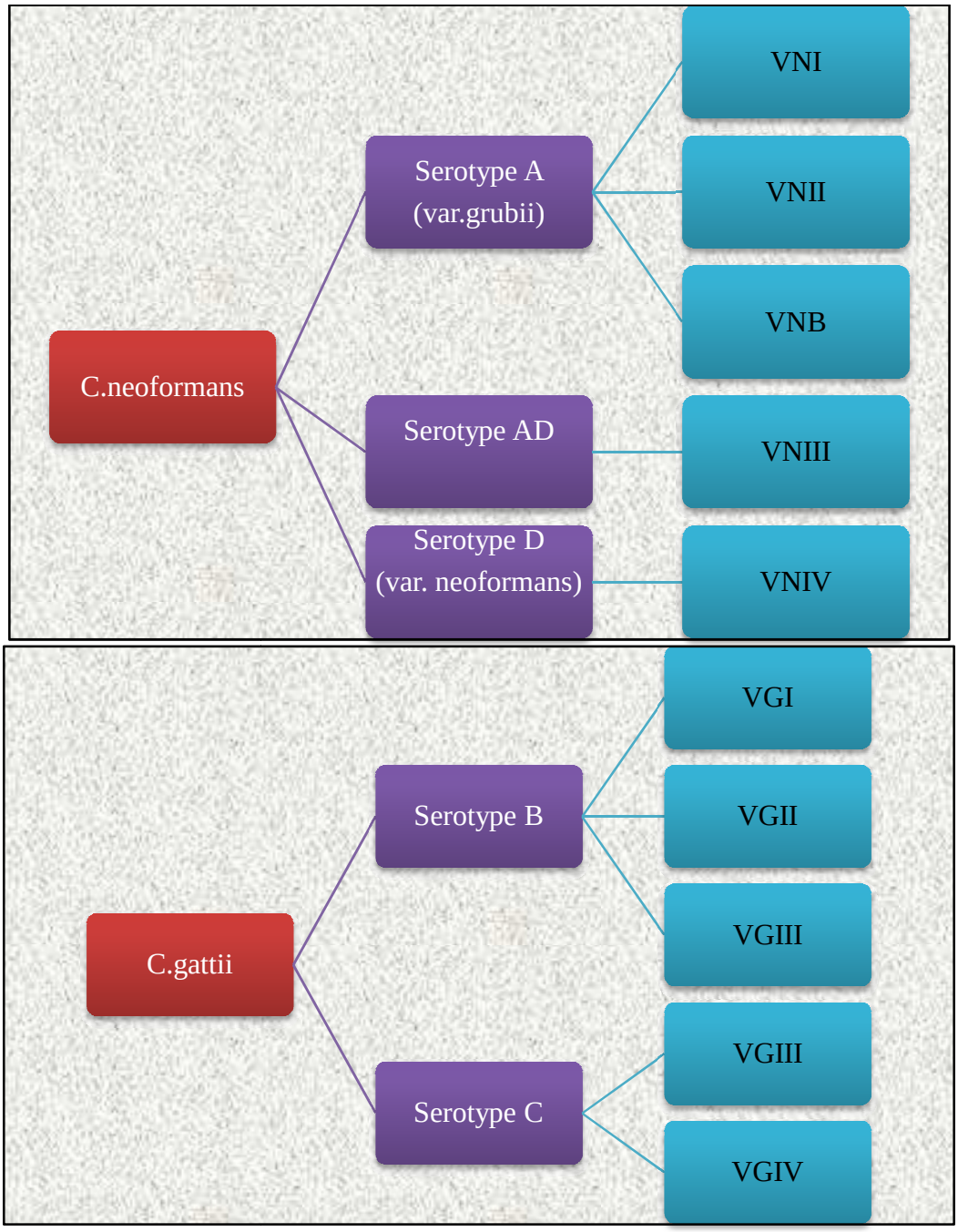
C.neoformans and the teleomorph was named *Filobasidiella neoformans*[51]. In 2003, the genome of *C.neoformans* was sequenced.

TAXONOMY

Cryptococcus genus is placed in the Fungi Kingdom under the Phylum Basidiomycota, Subphylum Basidiomycotina, Class Urediniomycetes, Order Sporidiales and Family Sporidiobolaceae. Traditionally 5 serotypes of *Cryptococcus neoformans* have been described based on variation in the capsular epitopes[75]. The serotypes are characterized using antibodies from rabbit sera or specific monoclonal antibodies. For several decades, serotypes A. D & AD were included under var. *neoformans* and serotypes B & C under var. *gattii*[75]. The varietal status was accorded on the basis of differences in biochemical tests. With the advent of molecular era it became evident that serotypes A and D significantly diverge from each other. Franzol *et al* in 1999 proposed to separate these serotypes into 2 varieties based on differences in URA5 gene sequences[30] (coding for orotidine monophosphate pyrophosphorylase). Using a variety of genetic analyses, Xu *et al* estimated that the A and D serotype separated from each other more than 18 million years ago[95]. Now serotype A has been distinguished as a new variety, var. *grubii* and serotype D has been retained under var. *neoformans*. The varietal status for AD serotypes is unclear but evidence states that they probably represent hybrids between serotypes A and D. In 2006 *Cryptococcus gattii* was accorded separate species recognition based on significant differences in the ecology and epidemiology. Therefore, *C.neoformans* is currently classified into 2 varieties (var.*grubii* and var. *neoformans*) and *C.gattii* is its sibling species[58].

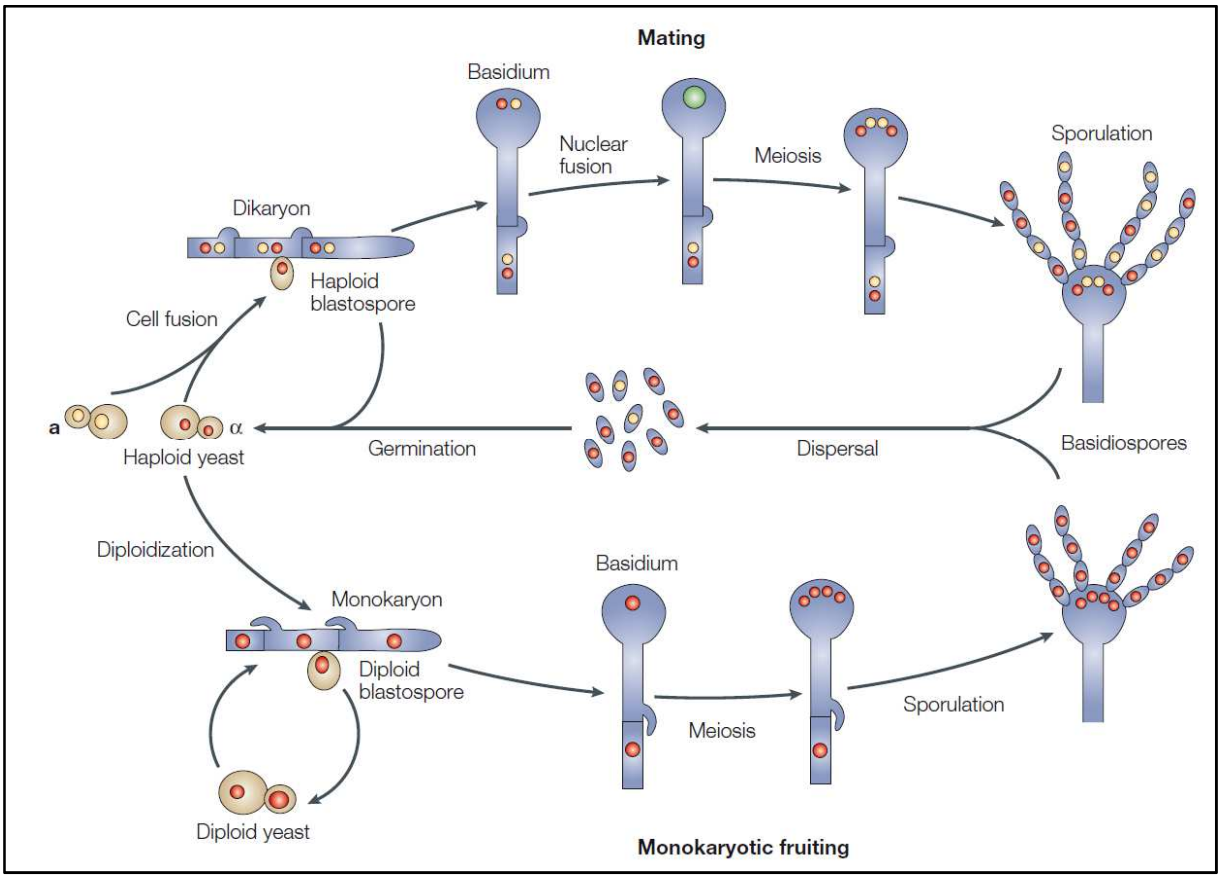
The taxonomic classification of these varieties and serotypes has been further updated through new genomic analysis. On the basis of DNA sequence polymorphisms

detected by PCR fingerprinting, RAPD, AFLP, RFLP and MLST analyses, *C.neoformans* and *C.gattii* have been further subdivided into 9 distinct molecular types[58]. Serotype A isolates produce VNI, VNII, or VNB (Botswana) pattern; AD hybrids produce VNIII pattern and serotype D isolates produce VNIV pattern. Serotype B isolates yield VGI, VGII and VGIII patterns while serotype C isolates yield predominantly a VGIII or VGIV pattern.



LIFE CYCLE

Cryptococcus neoformans can exist in two distinct life cycles- asexual and sexual[77]. In the asexual stage, it exists as encapsulated yeast cells and reproduces by simple, narrow based budding. These unicellular haploid yeasts are the primary forms recovered from both environmental sources and human infections. However, as a heterothallic basidiomycete, *Cryptococcus neoformans* can undergo transition to a hyphal growth form by 2 different differentiation mechanisms- Mating and Monokaryotic fruiting.



Yeast forms of *Cryptococcus neoformans* exist in one of the two mating types: a or α. Mating in *Cryptococcus* was discovered more than three decades ago by Kwon- Chung and involves fusion of haploid cells of opposite mating types to produce dikaryotic hyphae. Under nutrient limiting conditions, a and α yeast cells secrete peptide pheromones

that trigger cell- cell fusion. The resulting dikaryon initiates hyphal growth and a septum forms to separate the cells. These hyphae bear true clamp connections. During the formation of basidium, the 2 nuclei fuse and undergo meiosis to produce meiotic products. This results in the formation of 4 chains of basidiospores by mitosis and the uninucleate basidiospores bud off in a basipetal manner from the terminal portion of the basidium. The spores are initially unencapsulated but quickly develop capsule and begin budding as yeasts thus completing the sexual cycle. *C.neoformans* contains only one MAT locus and it occurs in 2 idiomorphic forms corresponding to the two mating types a and α. It has been observed that environmental and clinical isolates are predominantly of α mating type occurring in 30-40 fold higher magnitudes than the corresponding a mating counterparts[52].

Cryptococcus neoformans can undergo intra or inter varietal mating (serotype A x A, A x D, D x D) and the resulting teleomorph is called *Filobasidiella neoformans* var. *neoformans.* Mating in *C.gattii* (B x B, B x C, C x C) produces the teleomorph *Filobasidiella neoformans* var. *bacillispora*. The teleomorph of the two species can be distinguished on the basis of morphology[51]. *Filobasidiella neoformans* var. *neoformans* produces chains of spherical basidiospores whereas *Filobasidiella neoformans* var. *bacillispora* produces chains of oval/elliptical basidiospores. *Cryptococcus* can also undergo interspecies matings (B x D, A x C, C x D) but the viability of the basidiospores is reduced and many aneuploid progeny are generated indicating that genomic divergence impairs meiosis[58].

The mating of *Cryptococcus neoformans* has been observed in the laboratory under certain conditions like room temperature, minimal water exposure and environmental pCO_2 concentrations. Non enriched media such as V8 juice agar, hay infusion agar or bird guano filtrate agar are suitable for mating of *C.neoformans* in the laboratory.

Cryptococcus neoformans var. *neoformans* strains undergo monokaryotic fruiting, a modified form of sexual reproduction occurring between strains of the same mating type[52]. During this process, cells of one mating type eg., α become diploid α / α , either by endoduplication or by nuclear fusion following cell fusion between two α cells. The diploid monokaryotic hyphae form rudimentary clamp connections. At the stage of basidium development, meiosis occurs and haploid basidiospores are produced in four chains. Monokaryotic fruiting is predominantly observed in α strains and this might explain why α strains are more abundant[36].

ECOLOGY

Cryptococcus species are saprobes found in decaying wood, soil, fruits and vegetables[73]. It is now clear that *C.neoformans* and *C.gattii* reside in separate environmental niches. In 1955, Emmons first isolated *C.neoformans* from soil, pigeon droppings and pigeon nests[26]. The soils that most commonly yield *C.neoformans* are those which are frequented by pigeons, chickens, turkeys and to a lesser extent by canaries and parrots. Pigeons however seldom develop clinical disease due to *Cryptococcus*. This is explained by the fact that the average body temperature of pigeons is as high as 42^0C which allows survival in the gut but is not conducive to the growth of *C.neoformans*[77]. Since *C.neoformans* can metabolise creatinine which is abundant in bird guano, it is likely that there is a natural selection for *C.neoformans* to inhabit and grow in this niche. *C.gattii* unlike *C. neoformans* has never been cultivated from bird guano. The lack of *C.gattii* in the excrement of birds can be explained due to the high pH present in bird guano as *C.gattii* is more sensitive to pH changes than *C.neoformans*[76]. Ellis and Pfeiffer in 1990 cultured *C.gattii* from vegetation around river red gum trees (Eucalyptus camaldulensis) and forest red gum trees (E.tereticornis) in Australia[25, 79]. In recent years *C.gattii* has also been isolated from other gum trees such as flooded gum (E.rudis), blakely's red gum

(E.blakely), tallow wood (E.microcorys), turpentine gum tree (Syncarpia glomulifera) and pink shower tree (Cassia grandis). In Columbia, *C.gattii* isolates have been found in several new species of trees such as Terminalia catappa, Ficus soatensis, Croton bogotanus, Coussapoa spp, Cupressus lusitanica, Pinus radiata, and Acacia decurrens[58]. In India it is prevalent in Syzygium cumini trees[18]. In the 2002 outbreak of *C.gattii* on Vancouver Island, Canada, the fungus was recovered from alder, douglas fir, garry oak, arbutus and cedar trees[90]. These indicate that *C.gattii* is associated with panoply of potential host trees.

EPIDEMIOLOGY

Cryptococcus neoformans is prevalent worldwide with different geographic regions showing some differences in the strain prevalence. Serotype A is overwhelmingly prevalent over all other serotypes. Serotype D is generally restricted to European countries especially Denmark, France, Germany, Italy and Switzerland [75]. In a review of 725 clinical isolates of *Cryptococcus* by Kwon- Chung and Bennett [8], 80% of all isolates were *C.neoformans* and of these isolates 80% were *C.neoformans* var. *grubii*.

Majority of patients with disseminated cryptococcosis have an underlying immunocompromised condition. However, upto 20% of patients may not have any apparent underlying disease or risk factor (Pappas *et al* 2001)[77].

CONDITIONS ASSOCIATED WITH PREDISPOSITION TO CRYPTOCOCCUS NEOFORMANS INFECTION	
HIV infection	Organ transplantation
Lymphoproliferative disorders	Sarcoidosis
Haematological malignancies (CLL)	Corticosteroid therapy
Hyper IgM syndrome	Idiopathic CD4 Tcell lymphopenia
Treatment with monoclonal antibodies (Infliximab)	Peritoneal dialysis
Systemic Lupus Erythematosus	Cirrhosis

The incidence of cryptococcal infection peaked with the spread of the AIDS pandemic. Following widespread use of HAART, the incidence has shown a declining trend [3]. But still C*ryptococcus* continues to top the list of culture proven cases of meningitis in areas of the world with less access to ART.

The medical fraternity witnessed a rise in cryptococcal infections in solid organ transplant (SOT) recipients as a result of prolonged immunosuppression. In one study, cryptococcosis occurred in 2.8% of all infections encountered in SOT recipients[75]. The risk is greater for renal and liver transplant recipients when compared to bone marrow transplant recipients.

Increased use of corticosteroids has also contributed to the rise in the number of cryptococcal infections. Most of the affected patients in this cohort are infected with *C.neoformans* var. *neoformans* (serotype D).[77]

Cryptococcus gattii was generally considered to be restricted to tropical and subtropical areas such as Australia, Brazil, Central Africa, Hawaii, Southern California and South East Asia corresponding to the distribution of its ecological niche. However, an

outbreak of *C.gattii* on Vancouver Island has unraveled that the yeast is actually more widespread than was actually thought of. [7, 90] More than 160 human cases caused mainly by VGII genotype were reported and 6 proved to be fatal. Over the decades, *C.gattii* has been associated with infections in immunocompetent hosts and only rarely affects patients with AIDS. The reason why AIDS patients are less likely to develop *C.gattii* is not known. It has been hypothesized that since HIV is predominantly an urban infection, these patients may not be exposed to the ecological niches of *C.gattii*. It is also likely that *C.neoformans* produces dormant infections which are reactivated during HIV infection in contrast to *C.gattii* which may produce disease during the primary infection itself [77]. Of late, *C.gattii* is emerging as a pathogen even in immunocompromised hosts. It has been reported to cause 10% of cryptococcal infections in AIDS patients in Southern California and in Botswana and Malawi in Sub- Saharan Africa [58]. In India too, there are few recent reports stating the isolation of *C.gattii* from HIV patients [14, 71].

Cryptococcosis is predominantly a disease of adults with males being more commonly affected than females[76]. Children, however seldom seem to be affected by this yeast including those with AIDS[1, 76]. The explanation for the rarity of the disease in children still remains an enigma despite studies showing that most children acquire antibodies to *Cryptococcus* before the age of 10 (Chen *et al* 1999, Goldman *et al* 2001)[32]. It is interesting to note that several children with Hyper IgM syndrome tend to develop cryptococcal infections[75]. The exact mechanisms underlying this association need to be elucidated further.

Human to human transmission of *Cryptococcus* does not occur except in cases of transplantation of infected tissue [9](Beyt & Waltman 1978, Kanj *et al* 1996).

Although a wide range of animals and avians are infected by cryptococcal species, cryptococcosis has not been generally reported as a zoonosis. In 2000, Nosanchuk *et al* reported a single case of *C.neoformans* infection in a transplant recipient whose strain was identical to the one isolated from the cage of his pet cockatoo[72]. Except for this isolated report, there has been no documentation of such related transmissions so far. *Cryptococcus* affects both domestic and wild animals and the clinical presentation often differs from that in humans[58]. Cryptococcosis in cats presents mostly as upper respiratory tract infection. Dogs commonly develop severe disseminated disease with CNS and ocular involvement. Cryptococcal infection leads to mastitis and pneumonia in herds of cattle or goats. In horses, obstructive growths are seen in the nasal cavity and in koalas, asymptomatic nasal or skin colonization occurs. Despite the well known association of *C.neoformans* with pigeons for over 50 years, clinical disease is seldom encountered and they most probably serve as vectors in dissemination of the yeast[10].

PATHOGENESIS

Human infection is acquired primarily by inhalation of *Cryptococcus* into the lung alveoli. It is still unclear whether basidiospores or yeast cells are the infectious propagule of *C.neoformans*[58]. The ciliary action of respiratory epithelium removes particles >5µ, such as encapsulated yeast cells. Basidiospores, which are <2µ can ideally get lodged in the lung alveoli. However, yeast cells can get reduced to a size of 3µ or less by losing the capsule under conditions of nutrient deprivation and low moisture. But, basidiospores are more resistant than encapsulated yeast cells to dessication and readily become airborne and hence could more likely be the initiators of pulmonary infection. However, more studies are needed to confirm these hypotheses.

The ultimate presentation of cryptococcosis is a reflection of the complex interactions between the virulence of the infecting strain and host defence mechanisms. Once infection is established, one of the three possible processes could take place-

1. In an immunocompromised host, the yeast continues to proliferate and disseminate, causing clinical disease
2. In an immunocompetent host, effective immune response completely eliminates the yeast from the host
3. The yeasts produce a small lung/lymph node complex and remain dormant in tissues

The most important determinants of virulence in *Cryptococcus* include possession of a capsule, production of melanin and growth at 37^0C.

Capsule- The polysaccharide capsule is a major virulence factor for *C.neoformans* composed of unbranched chain of α-1, 3 linked mannose units substituted with xylosyl and β glucuronyl groups. About 80% of the capsule is made of glucuronoxylomannan while galactoxylomannan accounts for the remaining 20% [77].The serotypes of *C.neoformans* differ in the degree of mannosyl substitution, in the molar ratios of mannose, xylose and glucuronic acid and in the percentage of O-acetyl attachments. Mutant cryptococci that are hypocapsular or acapsular are less virulent in animal models than encapsulated strains[31]. Similarly, infection caused by poorly encapsulated strains is associated with less severe disease. The size of the capsule varies between strains and can account for >50% of the yeast diameter in some strains. Several physiological conditions like ambient pCO_2, presence of serum and limited availability of ferric ions increase the capsular size (Granger *et al*1985; Vartivarian *et al* 1993; Zargoza *et al* 2003). The capsular material can be shed from the yeast into body fluids such as blood and CSF. The capsular polysaccharide has the following effects on immunity: antiphagocytosis, decreases

antibody responsiveness, depletion of complement, inhibition of leucocyte migration, dysregulation of cytokine secretion[77]. Signaling pathways employing cAMP control the expression of capsule production and down regulation of this pathway produces an attenuated virulence phenotype.

Melanin production- *C.neoformans* and *C.gattii* are unique among other members of the genus in possessing a laccase. The enzyme laccase (phenol oxidase) catalyses the conversion of diphenolic compounds such as L- dopa, nor-epinephrine and epinephrine to quinones which rapidly autopolymerise to form melanin[75]. The gene encoding for laccase is present on the inner aspect of the cytoplasmic membrane of the yeast and laccase negative, albino mutants exhibit attenuated virulence in animal models[53]. The propensity of *Cryptococcus* to invade the CNS may partially be explained by its ability to degrade naturally occurring catecholamines to an antioxidant protecting the yeast when it is in the catecholamine rich CNS[77]. Other postulated reasons for the neurotropism include the probable presence of specific receptors on neuronal cells coupled with availability of nitrogen sources like asparagine and creatinine in the CSF[84]. Melanin accumulates in the cell wall and provides support to the cell wall, interferes with T cell responses and also protection from temperature changes and antifungal agents.

Growth at 37^0C- The pathogenic cryptococci like other pathogenic fungi grow well at 37^0C. Calcineurin and RAS signaling pathways are associated with the yeast's ability to grow at mammalian body temperatures [2](Alspaugh *et al* 2000). Presence of a vacuolar ATPase and the stress sugar, trehalose also enable the yeast to withstand host body temperature. Mutants of *C.neoformans* that cannot grow well at this temperature are less virulent even when they possess capsule and laccase activity (Kwon- Chung *et al* 1982). Higher body temperatures nearing 40^0C slow the growth rate of the strains with

production of aberrant budding patterns and pseudohyphal forms. Isolates of serotype B, C and D are more sensitive to growth inhibition at higher temperatures than serotype A[64].

Other factors linked to virulence include possession of phospholipase and urease enzymes, genes for superoxide dismutase, production of mannitol and mating strain α[75]. Mutants of phospholipase B gene (PLB1) are hypovirulent; so are mutants with loss of the gene for urease enzyme, URE1. Mannitol scavenges free radicals that may damage the yeast and also contributes to the formation of brain edema. A study by Neilson *et al* showed that α strains of *C.neoformans* are more virulent than congenic a strains and these α strains are also more likely to penetrate the CNS during co- infection with congenic a strains.

IMMUNOLOGY

The earliest innate immune response to the entry of infectious propagules of *Cryptococcus* in the lung is mediated by alveolar macrophages. Most primary infections of the lung are asymptomatic in immunocompromised individuals because they are readily contained by the immune system. Patients with intact immunity mount a Th1 cytokine (IL-2, INFγ, TNFα) response culminating in a granulomatous type of inflammatory lesion. Several studies have emphasized the role of CD4 and CD8 cells in containing the dissemination of cryptococci from the lungs to the CNS. The cause for neurotropism of *Cryptococcus* is not very clear but might also be related to the lack of inhibitory action of serum and that the CNS may provide a refuge for *Cryptococcus* from host immune responses[84]. The lack of an effective CMI in patients with AIDS culminates in rapid dissemination of yeast cells throughout the body and a relatively higher fungal burden in tissues when compared with immunocompetent patients. It has been observed that patients infected with *C.gattii* usually present with cranial nerve palsies and hydrocephalus. The

likely explanation is that these strains being less virulent might permit a robust immune response which results in an insidious presentation and focal neurological signs. Disseminated cryptococcosis in the immunocompromised host may be due to reactivation of latent infection or sometimes, a primary uncontained infection due to an ineffective immune system. The concept of reactivation of a dormant focus of cryptococcal infection is strengthened by studies in France showing that African ex-patriots who lived in Europe for many years prior to the development of disease possessed an infecting strain consistent with the genotype found in African strains[75].

Since cryptococcosis is a rare event despite the fact that cryptococci are widespread in the environment, it is likely that most primary infections in the general population are sub-clinical. Due to the immunologically inert nature of the polysaccharide capsule of *Cryptococcus*, little antibody response is elicited following infection and no satisfactory skin test has been developed so far. With increasing studies pointing to the role of humoral immunity in recovery from cryptococcosis, focus on this arena has started gaining a foothold. With encouraging results of formation of high antibody titres to *Cryptococcus* in mice immunized with a Polysaccharide- Tetanus Toxoid conjugate vaccine, the hope for a human vaccine has been elevated[77]. These antibodies may facilitate opsonisation and enhance the functions of Natural Killer cells. Besides, some studies have also shown that antibodies to glucosyl ceramide and melanin are targets of humoral immunity in addition to the capsule[74].

CLINICAL MANIFESTATIONS

Although the lung and CNS are the most common sites of infection, *Cryptococcus* can virtually disseminate and infect any organ of the human body[85]. Multiple site involvement is often noticed in the severely immunocompromised host.

Pulmonary cryptococcosis- The presentation of pulmonary cryptococcosis may vary from asymptomatic colonization to life threatening pneumonia with evidence of Acute Respiratory Distress Syndrome (ARDS) [46]. Moreover, patients with AIDS may also be co-infected with other pathogens like *Mycobacteria*, *Nocardia*, *Pneumocystis* or *Histoplasma* further complicating the clinical diagnosis. Symptomatic patients present with fever, chest pain, cough with expectoration of mucoid sputum and rarely haemoptysis. Immunocompromised hosts often present with a meningeal rather than pulmonary syndrome due to rapid dissemination of the yeast to the CNS. Hence, it has been postulated that isolation of *Cryptococcus* from the lung of a patient with high risk for dissemination must be followed by a lumbar puncture to rule out CNS infection even in the absence of symptoms [75]. It has been estimated that about 90% of AIDS patients with cryptococcal pneumonia present with concomitant CNS infection at the time of initial diagnosis[76].

CNS cryptococcosis- Cryptococcal involvement of the CNS often manifests in the form of subacute meningitis or meningoencephalitis albeit acute and chronic meningitis may occur. Patients generally present with headache, fever, lethargy, altered sensorium of few weeks duration[85]. Patients with *C.gattii* infection have a higher tendency to develop cryptococcomas and may present with signs of raised intracranial tension (ICT) like vomiting, cranial nerve palsies or hemiplegia[76]. Obstructive hydrocephalus ensues in some patients and can result in personality changes or dementia. Rarely, severely immunocompromised patients with cryptococcal meningitis can present with rapidly developing coma and death.

Cutaneous and mucocutaneous cryptococcosis- With rare exceptional cases, skin lesions in cryptococcosis herald an underlying disseminated infection and occur in about 10-15% of cases[22]. Direct inoculation of *Cryptococcus* into the skin following

trauma or laboratory accidents has also been reported[76]. *C.neoformans* can produce almost any type of skin lesion ranging from papules, plaques and vesicles to ulcers and draining sinuses[87]. Most of the lesions tend to involve the face, neck or the scalp. Some of the lesions might be mistaken for molluscum contagiosum, herpes or rarely carcinoma/ kaposi's sarcoma. In patients who are profoundly immunocompromised, skin lesions may take the form of an abscess or rapidly progressing cellulitis. Akin to the occurrence of co-infection at other sites, cutaneous cryptococcosis may be associated with other disseminated fungal infections of the skin. Hence, biopsy with proper histopathology and culture is essential to arrive at a correct diagnosis. Patients infected with serotype D strains of *C.neoformans* tend to have a higher rate of skin involvement than those infected with serotype A strains. Singh *et al* in 1997 observed that solid organ transplant recipients on tacrolimus therapy for immunosuppression had a higher ratio of skin and soft tissue infection relative to CNS infection[89]. This has been attributed to the anticryptococcal activity of tacrolimus at 37-39^{0}C but not at lower temperatures enabling the yeast to multiply in the skin. Mucocutaneous lesions are rarer when compared with skin lesions and present as nodules, granulomas or ulcers.

Osseous cryptococcosis- Bone is involved in upto 5% of disseminated cases of cryptococcosis. Cranial bones, vertebrae and bony prominences are often involved with a contiguous soft tissue abscess. Patients generally present with pain and swelling of the affected area for a long period.

Visceral cryptococcosis- In disseminated cryptococcosis any organ of the body may have foci of infection. Most commonly, the infection takes the form of granulomatous lesions which may be mistaken for neoplasia[84].

Ocular cryptococcosis is a relatively common event in patients with disseminated cryptococcosis. There are also anecdotal reports of eye being the portal of entry of *Cryptococcus* following traumatic inoculation[9] (Beyt & Waltman 1978, Granger *et al* 1986, Birkmann & Bennett 1988). The most common ocular manifestations are palsies and papilledema. Optic neuritis or endophthalmitis can result in severe visual loss. In some cases, compression of the ophthalmic artery secondary to raised ICT is responsible for loss of vision.

Infection of the prostate gland is yet again common in dissemination cryptococcosis and the prostate may serve to protect the yeast during antifungal therapy[57].

In rare instances, *Cryptococcus* may cause endocarditis, mycotic aneurysms, peritonitis, renal abscess, adrenal insufficiency, hepatitis, thyroiditis, breast abscess or genital ulcers[76].

LABORATORY IDENTIFICATION

A) MICROBIOLOGICAL TECHNIQUES:

Although CSF in CM is usually clear and can be viewed directly under the microscope, sputum and pus samples must be digested in 10% KOH mount before performing a microscopic examination.

WET MOUNTS- The yeasts of *C.neoformans* are quite fragile and collapse in dried, fixed or stained films. Hence wet preparations are ideal for rapid identification of *Cryptococcus* in the laboratory. When the infected material containing cryptococci is mixed with a drop of India ink, 10% Nigrosin or other colloidal mounting medium, the encapsulated yeast cells of *Cryptococcus* (5-10µ) are delineated by negative contrast. Some amount of expertise is needed to differentiate the yeast cells from WBCs, RBCs or

talc granules that may have dropped from the gloves while preparation of the mount. The sensitivity of an India ink preparation is usually $\geq$75% in AIDS patients and only upto 50% in non- HIV infected patients with cryptococcal meningitis[54]. Usually, a concentration of yeast $\geq 10^4$ CFU /ml of CSF is necessary for detection by India ink preparation. A modified India ink preparation using 2% mercurochrome (Rito Zerpa *et al*, 1996) allows identification of external and internal structures of *Cryptococcus* with clear distinguishing of 3 layers of the capsule [96]. Internal corpuscles corresponding to different sizes of endogenous spores of the yeast are visible clearly in the modified preparation. 3 layers of the cryptococcal capsule namely lucent stratum, fibrillar material and light zone are easily made out in these preparations which are otherwise discerned only under the electron microscope.

GRAM STAIN- A Gram stain performed on sediment from a cytospin preparation of CSF shows the presence of pus cells and gram positive budding yeast cells.

CALCOFLUOR WHITE (CFW) STAIN- Can be used to identify *Cryptococcus* in cases where the number of yeast cells is expected to be scanty.

CULTURE- CSF sample must be inoculated on Sabouraud Dextrose Agar (SDA) with antibiotics but without cycloheximide as it inhibits the growth of *Cryptococcus*. Cultures must be incubated at 37^0C and 25^0C for a period of 4 weeks before declaring as sterile[27]. The yield of a positive culture is increased by culturing large volumes of CSF (10-12ml). Yeast like, mucoid, cream to buff coloured colonies of *Cryptococcus* generally appear after 2-3 days but may be delayed for over a week in case of patients on antifungal therapy. Rare, acapsular variants may produce dry or glabrous colonies[84]. An LCB mount from the colonies would show the presence of budding yeast cells. A negative culture does

not rule out cryptococcosis since the CSF is sterile in cases of cryptococcomas or in instances where very few organisms might have been present in the CSF.

GROWTH ON SPECIAL MEDIA:

1. CORN MEAL AGAR- *Cryptococcus* grows as yeast cells that exhibit narrow based budding without production of pseudohyphae.
2. STAIB'S AGAR- On Staib's agar containing extract of niger seed (Guizotia abyssinica), *C.neoformans* and *C.gattii* produce brown coloured, mucoid colonies by production of melanin from the substrates using the enzyme, phenol oxidase[16]. Other media employing the same principle are Pal's sunflower seed agar (containing Helianthus annuus) and a synthetic medium made of caffeic acid containing ferric citrate. Nardelli *et al* (2004) developed a Minimal Synthetic Caffeic Acid Medium (MSCAM) containing only caffeic acid, ferric citrate and noble agar without glucose, vitamins or mono/bivalent ions. They observed rapid pigment production and concluded that absence of glucose or Ca^{++} or NH_4^+ does not interfere with the activity of phenol oxidase. Vanessa *et al* (2005) modified Staib's medium without creatinine and observed rapid production of intensely pigmented brown colonies by *C.neoformans*.
3. CANAVANINE- GLYCINE- BROMOTHYMOL BLUE (CGB) AGAR- CGB medium is used to differentiate *C.neoformans* from *C.gattii*. The latter is resistant to the action of L-canavanine and can utilize glycine as the sole source of nitrogen producing a cobalt blue colour change[16]. Glycine Cycloheximide Phenol red agar (GCP) is also based on the principle of ability of *C.gattii* to utilize glycine.
4. CREATININE- DEXTROSE- BROMOTHYMOL BLUE- THYMINE (CDBT) AGAR- Used to differentiate *C.neoformans* var. *grubii* and var. *neoformans*. The latter will assimilate thymine and grow as red coloured colonies on this medium[38].

BIOCHEMICAL TESTS:

1. UREASE TEST- Members of the genus *Cryptococcus* have the ability to hydrolyse urea which could be detected on Christensen's urease medium or even within 10-30mts using a rapid urea broth. Using a modified urease assay, Kwon- Chung in 1987 demonstrated that the urease activity of *C.gattii* is suppressed following chelation with 100µM EDTA while that of *C.neoformans* is not[77]. Robert *et al* used 1% benzalkonium chloride with urea agar base for presumptive identification of *Cryptococcus* within 10-15 minutes[97].
2. NITRATE REDUCTION- *C.albidus* and *C.terreus* can reduce nitrate to nitrite.
3. NITRATE ASSIMILATION- *C.laurentii* is the species of *Cryptococcus* that assimilates nitrogen.
4. ASSIMILATION OF AMINO ACIDS- Used to differentiate *C.neoformans* from *C.gattii* as the latter can use D- proline[24] or D- tryptophan as the sole source of nitrogen.
5. ASSIMILATION OF CARBOHYDRATES- All cryptococci are non-fermentative and vary in their ability to assimilate carbohydrates[77, 84].

Cryptococcal species	Carbon assimilation					
	Inositol	Dextrose	Lactose	Maltose	Melibiose	Sucrose
C.neoformans and its var.	+	+	-	+	-	+
C. albidus and its var.	+	+/w	+/-	+	+/v	+
C.laurentii and its var.	+	+	+	+	+/-	+

C. luteolus	+	+	-/w	+	+/w	+
C.melibiosum	+	+	w	-	+	-
C.terreus	+	+	+/w	V	-	-

Note: + denotes sugar is assimilated, - denotes sugar is not assimilated, v- variable, w- weak assimilation of sugar

IMMUNODIAGNOSIS- The polysaccharide antigen of cryptococcal capsule is shed from the surface of the yeast cells and may be detected in serum, CSF, urine or rarely BAL[94]. The test is performed using commercially available Latex agglutination kits (LAT) or more recently by Lateral flow assays (LFA) employing immunochromatographic principles[53]. These tests are highly sensitive and can detect even 10ng/ml of the polysaccharide in the specimen. A cryptococcal antigen titre $\geq$ 1:8 is generally regarded as significant. The titre is highest in serum followed by CSF and urine[75]. False positive results may occur in upto 15% of the cases especially at titres $\leq$1:32. These might be due to the presence of rheumatoid factor in serum or in cases of infections with organisms that share cross reacting antigens like *Trichosporon*, *Capnocytophaga* or *Stomatococcus* and can be eliminated by pre-treatment of the serum with pronase or 2- mercaptoethanol[76]. False negative results on the other hand may result from prozone effect especially in serum samples and may be overcome by diluting the sample. Sujatha *et al* (2003) compared commercially available LAT with in- house Co-Agglutination (Co-A) test in 150 cases of chronic meningitis for detection of cryptococcal antigen in CSF. They

reported that Co-A test is inexpensive and useful adjuvant to microscopy and culture in diagnosing CM.

Detection of antibodies to *Cryptococcus* may be performed using agglutination, indirect fluorescent antibody test, complement fixation tests or EIA. However, these are not very reliable due to frequent occurrence of cross reactions with other diseases.

Serotyping techniques used in the past employed slide agglutination techniques using eight factors or five factors sera prepared by adsorption of rabbit polyclonal antisera[75]. These are unique to a particular serotype (factor 5- serotype B, factor 6- serotype C, factor 7- serotype A, factor 8- serotype D) or shared by two or more serotypes (factor 1- A, B, C, D; factor 2- A, B, D; factor 3- A, D; factor 4- B, C). A positive agglutination titre is generally 1:8 and above. Similarly, dot immune assays with serotype specific antisera were also in vogue. In addition, commercially available EIAs employing monoclonal antibodies can also differentiate the serotypes.

MOLECULAR METHODS- The species and serovar of cryptococcal strains may be determined by PCR fingerprinting using $(GACA)_4$, $(CAC)_5$ and FM1 primers. Species specific DNA probes using ribosomal ITS sequences have also been developed[58]. In Restricted Fragment Length Polymorphism (RFLP) analysis, *C.neoformans* repetitive element 1 (CNRE-1) hybridizes effectively with DNA of serotype A yielding 11-16 bands while 5-11bands are obtained with serotype D due to poor hybridization[66]. Multi Locus Enzyme Electrophoresis (MLEE) also produces serotype specific patterns such as ET1-4 (A), ET8-12 (D) and ET13-19 (B &C). Electrophoretic karyotyping by contour- clamped homogenous electrophoresis relies on the differences in the size and number of chromosomes of *Cryptococcus*. The number varies from 12.8 in *C.neoformans* var. *neoformans* to 13 for *C.gattii* and 121 for *C.neoformans* var. *grubii*. Random

Amplification of Polymorphic DNA (RAPD) analysis using mini-satellite specific core sequence of the wild type phage M13 (5'GAGGGTGGCGGTTCT3') as a single primer followed by amplification effectively identifies the genotypes of *Cryptococcus*. The mating type of *C.neoformans* can be determined using MLST techniques[58].

IN- VITRO ANTIFUNGAL SUSCEPTIBILITY TESTING- The reference broth dilution method recommended by Clinical and Laboratory Standards Institute (CLSI) is ideal for determining the MIC of antifungal agents to *Cryptococcus*[20]. The methodology has been standardized for media, inoculum and end point determination to test antifungal agents against *Cryptococcus* spp. However, E test may also be used to determine the MIC of antifungal agents to *Cryptococcus.* Commercially available automated systems like Vitek-2 can also be used for identification as well as MIC determination of *Cryptococcus*.

B) <u>OTHER ANCILLARY TESTS:</u>

1. **BIOCHEMICAL EXAMINATION OF CSF-** WBC cell count is generally elevated with mononuclear cell predominance (>50/µl) but may be low in patients with AIDS[54]. Glucose levels are slightly reduced and a modest rise in the level of proteins is observed. However, all parameters may be normal in severely immunocompromised patients[76].

2. **HISTOPATHOLOGICAL EXAMINATION OF BIOPSY SPECIMENS-** Presence of budding yeast cells in tissues surrounded by empty spaces can be observed using Periodic Acid Schiff (PAS), Haematoxylin and Eosin (H&E) or Gomori Methenamine Silver (GMS) stains. Rarely, aberrant mycelial forms may also be observed. Detection of the capsule using Alcian blue or Meyer's Mucicarmine is very helpful in the diagnosis by staining it blue or pink respectively[75]. For rare,

acapsular variants, Masson- Fontana stain must be employed to demonstrate the production of melanin by the yeast[76].

3. **RADIOLOGY-** Varied lesions may be present in chest roentgenogram of patients with pulmonary cryptococcosis including nodules, local/diffuse infiltrates, hilar lymphadenopathy and rarely, cavitation or pleural effusion[46].

Patients with cryptococcal meningoencephalitis can have normal CT and MRI scans on imaging. However, CT may reveal single/multiple nodules suggestive of cryptococcomas and also presence of hydrocephalus. MRI scans too may show hyperintense clustered foci on T2WI and non-enhancing on postcontrast T1WI in the basal ganglia or midbrain[75].

X-ray in osteomyelitis caused by *Cryptococcus* generally reveals one or more, well circumscribed osteolytic lesions and the absence of periosteal proliferation is indicative of cryptococcosis rather than other mycoses[84].

4. **ANIMAL PATHOGENICITY TESTING-** Suspected clinical specimen or a suspension of young culture is inoculated into swiss albino mice. The animals generally die in 7-10 days and yeast cells can be demonstrated in various visceral organs and body fluids[76].

MANAGEMENT

Cryptococcal meningitis is almost uniformly fatal unless appropriate antifungal therapy is instituted. Based on data analysed from several large scale studies, the current WHO recommendations for treatment of an episode of CM is a 2 phase approach- Induction followed by Consolidation[82]

Induction phase- Amphotericin B deoxycholate is administered at a dose of 0.7mg/kg/day in combination with flucytosine 100mg/kg/day for a period of 2 weeks. Liposomal amphotericin B 4mg/kg/day may also be used with reduced toxicity.

Consolidation phase- Fluconazole is administered at a dose for 400-800mg/day for 8 weeks

Suppressive prophylaxis- Tab. fluconazole is administered daily at a dose of 200mg/day. Discontinuation of anti-fungal maintenance treatment is recommended based on the following criteria[82]

a. <u>If HIV viral load monitoring is not available</u>

When patients are stable and adherent to ART and anti-fungal maintenance treatment for at least one year and have a CD4 cell count of greater than or equal to 200 cells/µl (two measurements six months apart).

b. <u>If HIV viral load monitoring is available</u>

Patient is stable and adherent to ART and anti-fungal maintenance treatment for at least one year and with a CD4 cell count of greater than or equal to 100 cells/ µl (two measurements six months apart) and a suppressed viral load.

Maintenance treatment for cryptococcal disease should be restarted if CD4 count drops to <u>≤</u>100 cells/ µl or if a WHO stage 4 clinical event occurs

Relapse in a case of cryptococcal meningitis is defined when an episode of CM occurs with the following characteristics[40]- (1) previous laboratory confirmed case of cryptococcal meningitis (2) recurrence of symptoms of meningitis (3) CSF India ink, antigen test (CRAG) and/or culture positive for *Cryptococcus* at this presentation (4) no alternative diagnosis. These relapse episodes can occur in patients who fail to take secondary fluconazole prophylaxis or even in patients on fluconazole prophylaxis before

the institution of HAART. A patient can present with more than one episode of relapse if there is resolution of symptoms and atleast one month between episodes. Sometimes, a relapse episode could be due to Immune Reconstitution Inflammatory Syndrome (IRIS) [40]. It is defined as (1) microbiologically confirmed first episode of CM (2) resolution of CM symptoms before starting ART (3) self-reported adherence to fluconazole and ART (4) recurrence of symptoms of meningitis after initiation of ART (5) no alternative diagnosis, including fluconazole resistance, found on laboratory testing and clinical review. Immediate ART initiation is not recommended in HIV-infected patients with cryptococcal meningitis due to the high risk of IRIS which may be life-threatening [69, 88]. WHO guidelines state that ART initiation should be deferred until there is evidence of a sustained clinical response to anti-fungal therapy [82], and usually after 2-4 weeks of induction and consolidation treatment with amphotericin B combined with flucytosine or fluconazole. [43]

Supportive measures- In patients with very high intracranial pressures, repeated lumbar punctures with a wide bore needle may be needed[33, 60]. Additionally, optic nerve fenestrations might be required to prevent visual loss due to the effects of raised ICT. A ventriculo-peritoneal shunt procedure is often necessary in patients with non-communicating hydrocephalus. Surgery for cryptococcomas especially those >3cm needs to be individualized on patient basis after administering adequate antifungal therapy[75]. Finally, the role of cytokines in accentuating the killing of cryptococci has been evaluated in few prospective human trials. Treatment with INF-γ had a remarkable reduction in yeast counts in the CSF but had little impact on the final outcome of the patients. More research needs to be done on the use of immunomodulators in the management of cryptococcosis[58].

PROGNOSTIC FACTORS

The nature of the underlying illness of the patient is the most important determinant of the response to anticryptococcal therapy. Patients with advanced neoplasia or AIDS generally have a poor prognosis. Several studies have implicated that mortality rates are higher in patients with cryptococcal meningitis who exhibit the following features[23, 62, 98]

1. High opening pressure on lumbar puncture (>250mm of H_20)
2. Higher yeast burden at the time of presentation as detected by India ink or elevated capsular polysaccharide antigen titre $\geq$1:1024
3. Poor inflammatory response indicated by the paucity of WBCs in the CSF < $20/mm^3$

Further, the presence of headache is considered as a good prognostic factor as the patient generally seeks early diagnosis and treatment[77]. On the contrary, altered mental status or stupor is indicative of poor prognosis.

PREVENTION

The use of prophylactic fluconazole therapy for preventing opportunistic fungal infections in AIDS patients with low CD4 counts has resulted in measurable decline in the incidence of cryptococcosis. In the pre- HAART era, 50-60% of patients relapsed after therapy was stopped but relapse rate reduced to 5% after introduction of suppressive therapy[13]. However, the threat of development of drug resistance remains a major concern.

In 1991, a GXM-TT conjugate vaccine was found to be protective in mice but human studies in patients with risk for cryptococcal infection are still lacking[77]. Few

reports also point to the role of serotherapy using poly or monoclonal antibodies but further studies are needed to define their clinical effectiveness.

High risk patients may also be refrained from entering sites with abundant aerosolized pigeon droppings as a measure to prevent infection.

Materials and methods

MATERIALS AND METHODS

ETHICAL CONSIDERATION- Approval by the Institutional Ethics Committee, Madras Medical College and Institutional Review Board, Government Hospital of Thoracic Medicine was obtained prior to the commencement of the study. All patients satisfying the inclusion criteria were included in the study. Informed consent was obtained from the patients/ relatives (in case of patients with altered sensorium) and a structured questionnaire was used to obtain details of the patient.

PLACE OF STUDY- The study was conducted on HIV positive patients admitted to the medicine wards of either of the two hospitals

1- Rajiv Gandhi Government General Hospital, Chennai-3

2- Government Hospital of Thoracic Medicine, Tambaram, Chennai- 47

DURATION OF STUDY- The study was conducted over a period of one year between October 2011to September 2012

INCLUSION CRITERIA- Patients infected with HIV and presenting with symptoms suggestive of meningitis like

1. Fever
2. Diffuse headache
3. Nausea/vomiting
4. Drowsiness/ altered sensorium
5. Visual disturbances: blurring/ diplopia (or) photophobia

EXCLUSION CRITERIA-

1. HIV negative patients presenting with features suggestive of meningitis

COLLECTION OF SAMPLES- Under strict aseptic precautions, samples were collected from the patients and transported as soon as possible to the laboratory. Cerebrospinal fluid (CSF) and blood was collected from all patients included in the study. In addition; depending on the symptoms, sputum, touch smears of skin lesions, tissue biopsy or other samples were collected from patients presenting with extrameningeal involvement besides meningitis.

COLLECTION OF CSF- Patient was placed in left lateral position. The spine was flexed and lumbar vertebrae L4 and L5 were identified. The skin was disinfected with 70% alcohol and 10% povidone iodine. A spinal needle was introduced into L4-L5 intervertebral disc space and about 3-5 ml of CSF was collected separately in three leak proof, sterile, screw capped containers- first one for biochemical analysis, next one for microbiological techniques and the last for cytology[54].

COLLECTION OF BLOOD SAMPLE- The venepuncture site was thoroughly disinfected with 70% alcohol for 30 seconds followed by betadine for 30 seconds to 1 minute. The disinfection was done in circular movements in a unidirectional manner from the centre to periphery and 2ml of blood was collected in a vacutainer containing EDTA[54].

BIOSAFETY CONSIDERATIONS- In view of the infectious nature of the samples, all the procedures were carried out in a biosafety level 2 cabinets. Adequate protective equipments were used while performing the tests. The samples were disinfected in freshly prepared solution of 1% sodium hypochlorite for 30 minutes before disposal.

PROCESSING OF SAMPLES- CSF was centrifuged at 1500-2000 rpm for 10-15 minutes and the deposit was used for performing India ink preparation, Gram stain and culture. The supernatant was used to perform LAT. In case of delay in processing, the CSF sample was incubated at 37^0C till further processing.

INDIA INK PREPARATION- A loopful of centrifuged CSF sediment was placed on a clean glass slide and a drop of India ink was added to the slide. The sediment and India ink were mixed thoroughly with a sterile needle and a coverslip was carefully placed over the mixture avoiding air bubbles[54]. The preparation was viewed under 10X and 40X objectives of the microscope to look for presence of encapsulated, spherical, budding yeast cells. The edge of the coverslip was specifically examined as the yeast cells tend to move towards the periphery along with the fluid while placing the coverslip. In addition, the India ink was regularly checked for contamination by examining just the stain under the microscope.

CSF samples with an ambiguous capsule were re-examined with a modified India ink preparation containing 2% mercurochrome[96]. A small drop of centrifuged CSF deposit was placed on a clean glass slide and a small drop of 2% mercurochrome was then mixed with the CSF on the slide. Then, a small amount of India ink was immediately added and a cover slip was placed without further mixing. The preparation was viewed under 10X and 40X objectives of the microscope to appreciate the 3 layers of the capsule of cryptococcus. A clearly layered halo around the yeast cell against a pinkish-black background could be appreciated.

GRAM STAIN- A clean glass slide was taken and a loopful of sediment placed on it to make a smear. The smear was air dried and fixed by flaming the slide. The smear was covered with methyl violet and left undisturbed for 1 minute. The slide was tilted to drain

the methyl violet and rinsed in flowing tap water. The smear was then covered with Gram's iodine for 1 minute and rinsed in the same manner. Smear was decolourised with acetone for 1-2 seconds and quickly rinsed with water. The smear was finally covered with dilute carbol fuschin for 1minute and rinsed similarly[54]. The stained smear was air dried and observed under 100X oil immersion objective for the presence of gram positive, budding yeast cells.

LATEX AGGLUTINATION TEST (LAT) - The supernatant from the centrifuged CSF sample was inactivated by placing in a boiling water bath for 5 minutes to reduce non-specific interference in the test. The kit used was Cryptococcal Antigen Latex Agglutination System (CALAS), [Meridian Biosciences]. The following procedure was followed-

1. One drop of positive control was added to each of the two designated rings.
2. 25 µl of the antibody control and negative control were added to the appropriate rings.
3. 25 µl of CSF sample was added to each of the two designated rings.
4. One drop of detection latex was added to each of the designated rings.
5. Similarly, one drop of control latex was added to each of the designated rings.
6. The contents of the rings were mixed using separate applicator sticks.
7. The card was rotated at 125 rpm for 5 minutes on a rotator and the reaction was graded as follows:

a) Negative: homogenous suspension of particles with no visible clumping
b) 1+ : fine granulation against a milky background
c) 2+: small but definite clumps against a slightly cloudy background
d) 3+: large and small clumps against a clear background
e) 4+ large clumps against a very clear background

A reaction of 2+ or more with the detection latex was taken as positive for the presence of cryptococcal polysaccharide antigen in the CSF

CULTURE- CSF sample was inoculated on two slants of Sabouraud Dextrose Agar (SDA) and incubated at 37^0C and 25°C respectively. The inoculated slants were inspected daily during the first week and twice weekly for another 3 weeks before discarding as negative[27].

Appearance of moist, mucoid, cream coloured colonies on SDA following 48-72 hours of incubation was presumptively identified as *Cryptococcus* and further tests were done to confirm the identity of the isolate.

GRAM STAIN: A Gram stain was performed on any yeast grown on SDA to ascertain the presence of gram positive, budding yeast cells.

UREASE TEST: Detection of urease activity of *Cryptococcus* was performed using Christensen's urease medium. Using a loop, a small amount of pure growth from a 48-72hr old culture was inoculated on urease slants and the tubes were incubated at 25°C. If urease is present in the isolate, urea is split to ammonia which raises the pH turning the slant to deep pink colour due to the presence of phenol red indicator[16]. The urease test was declared negative only after 4 days. ATCC *C.neoformans* 32045 and ATCC *C.albicans* 90028 were used as positive and negative controls respectively.

GROWTH ON CAFFEIC ACID FERRIC CITRATE (CFA) MEDIUM: 2-3 colonies of pure growth from a 48-72hr old culture were inoculated on CFA medium and the plates were incubated in dark at 25°C for upto 5 days. The appearance of dark brown colonies is interpreted as positive for melanin production[29]. ATCC *C.neoformans* 32045 and ATCC *C.albicans* 90028 were used as positive and negative controls respectively.

GROWTH ON CANAVANINE- GLYCINE- BROMOTHYMOL BLUE (CGB) AGAR: 1-2 colonies of pure growth from a 48-72hr old culture were streaked on the surface of CGB slants using a straight wire loop and incubated at 25°C for upto 5 days. Development of a cobalt blue colour in the medium due to the rise in pH indicates that the isolate grown is *C.gattii*. The glycine in the medium is metabolised to ammonia by *C.gattii* which raises the pH to 7 from 5.8 changing the colour to blue[16]. An in-house *C.*gattii (serotype B) and ATCC *C.neoformans* 32045 were used as positive and negative controls respectively.

GROWTH ON CREATININE- DEXTROSE- BROMOTHYMOL BLUE-THYMINE (CDBT) AGAR: 1-2 colonies of pure growth from a 48-72hr old culture were inoculated on CBDT medium and the plates were incubated at 25°C for upto 5 days. Growth of red colonies is indicative of assimilation of thymine by *C.neoformans* var. *neoformans*. *C.neoformans* var. *grubii* fails to grow on this medium due to its lack of ability to assimilate thymine[38]. ATCC *C.neoformans* 32045 (var. grubii) was used as negative control.

ASSIMILATION OF CARBOHYDRATES- The pour plate auxanographic method of Wicherham and colleagues was used [28, 56]. For testing each isolate, 90ml of agar (2%) was autoclaved at 121^0C for 15 minutes. 0.67 grams of Yeast Nitrogen Base (YNB) was added to 5ml of distilled water and sterilized by filtration. Heavy yeast suspension made from 4-5 colonies of the 48-72 hr culture was added to 5ml of YNB prepared. It was emulsified to turbidity equal to 4 McFarland standard. The YNB & yeast suspension was added to 90ml of molten agar cooled at 45°C, gently swirled to distribute the yeast cells in the molten agar, and the entire volume was poured into a 150mm petri plate. Sugar discs were placed in a circle with sterile forceps, such that at least 30mm is present between centers of each disc and incubated at 25°C for 3-4 days. The discs used were inositol,

dextrose, cellobiose, lactose, maltose and sucrose. Appearance of growth around a sugar disc indicates assimilation of that sugar by the test isolate. Growth around dextrose disc was recorded first to signify the viability of the yeast. ATCC *C.neoformans* 32045 was used as control stain which assimilates dextrose, maltose and sucrose strongly. Inositol assimilation is delayed but positive while cellobiose is weakly assimilated. Lactose, however, is not assimilated.

ASSIMILATION OF PROLINE- 11.7 grams of Yeast Carbon Base (YCB) was dissolved in 100ml of distilled water and sterilized by filtration. 20 grams of agar was dissolved in 980 ml of distilled water and dispensed as 18ml each in 18x 150mm screw capped test tubes and autoclaved at 121^0C for 15 minutes. Yeast suspension was made by emulsifying 2-3 colonies of a 48-72 hr culture in 2ml of prepared YCB to obtain turbidity equal to 1 McFarland standard. The yeast suspension was added to 18ml of molten agar cooled at 45°C, gently swirled to distribute the yeast cells in the molten agar, and the entire volume was poured into a 90mm petri plate. A proline disc was placed with sterile forceps and incubated at 25°C for 7 days[24]. Presence of growth around the proline disc indicates assimilation of proline by the yeast. *C.gattii* assimilates proline while *C.neoformans* does not. Appropriate control strains were similarly tested.

ANTIFUNGAL SUSCEPTIBILITY TESTING- Determination of Minimum Inhibitory Concentration (MIC) was done by microbroth dilution method as per CLSI M27-A3 protocol[20]. In addition, Epsilometer test was performed to compare its performance with the recommended microbroth dilution method.

MICROBROTH DILUTION METHOD:

Amphotericin B powder was obtained from Himedia, Mumbai whereas voriconazole and fluconazole powders were obtained from Pharma Fabricon. Their potency was 750µg/mg each.

$$\text{Weight (mg)} = \frac{\text{volume (ml) x desired concentration (µg/ml)}}{\text{Assay potency (µg/ml)}}$$

$$\text{Volume (ml)} = \frac{\text{weight (mg) x assay potency (µg/ml)}}{\text{Concentration (µg/ml)}}$$

STOCK SOLUTION:

For water soluble drugs like fluconazole sterile water was used as solvent, whereas for water insoluble drugs like amphotericin B and voriconazole, dimethyl sulfoxide (DMSO) was used. Stock solution of 5200µg/ml for fluconazole and 1600µg/ml for the other two drugs was prepared. The drugs were diluted from the stock solution in RPMI (fluconazole) or DMSO (amphotericin B and voriconazole) to obtain an intermediate solution. This was then further diluted 1:10 (fluconazole) or 1:100 (amphotericin B and voriconazole) in RPMI to obtain the final strength. This procedure was done to avoid dilution artifacts that result from precipitation of compounds with low solubility in aqueous media.

TEST MEDIUM USED:

Rosewell Park Memorial Institute (RPMI) 1640 (with glutamine, without bicarbonate, phenol red as pH indicator) [Himedia, Mumbai]

INOCULUM PREPARATION:

All *C.neoformans* isolated from patients and ATCC *C.neoformans* were subcultured on Sabouraud Dextrose Agar and incubated at 35^{o}C for 48hours to ensure purity and viability. The control strains, ATCC *Candida krusei* and ATCC *C.parapsilosis* were incubated for 24 hours only. Colonies were suspended in 5ml of sterile saline and adjusted to match turbidity of 0.5McFarland standard. The resulting yeast stock suspension of $1x10^6$ - $5 x10^6$ cells/ml was converted to working suspension by diluting it with RPMI 1640 broth medium in 1:100 followed by 1:20 dilution resulting in $5x10^2$ - $2.5x10^3$ cells/ml.

PROCEDURE

Scheme for preparing dilution series of water soluble antifungal agents

Antimicrobial solution						
Step	Concentration (µg/ml)	Source	Volume (ml)	RPMI Medium (ml)	Intermediate concentration (µg/ml)	Final concentration at 1:10 (µg/ml)
1	5120	stock	1	7	640	64
2	640	step 1	1	1	320	32
3	640	step 1	1	3	160	16
4	160	step 3	1	1	80	8
5	160	step 3	0.5	1.5	40	4
6	160	step 3	0.5	3.5	20	2
7	20	step 6	1	1	10	1
8	20	step 6	0.5	1.5	5	0.5
9	20	step 6	0.5	3.5	2.5	0.25
10	2.5	step 9	1	1	1.25	0.125
11	2.5	step 9	0.5	1.5	0.625	0.0625
12	2.5	step 9	0.5	3.5	0.3125	0.03125

Scheme for preparing dilution series of water insoluble antifungal agents

Antimicrobial solution						
Step	Concentration (µg/ml)	Source	Volume (ml)	Solvent (ml) Eg. DMSO	Intermediate concentration (µg/ml)	Final concentration at 1:100 (µg/ml)
1	1600	stock			1600	16
2	1600	stock	0.5	0.5	800	8
3	1600	stock	0.5	1.5	400	4
4	1600	stock	0.5	3.5	200	2
5	200	step 4	0.5	0.5	100	1
6	200	step 4	0.5	1.5	50	0.5
7	200	step 4	0.5	3.5	25	0.25
8	25	step 7	0.5	0.5	12.5	0.125
9	25	step 7	0.5	1.5	6.25	0.0625
10	25	step 7	0.5	3.5	3.13	0.0313

DMSO = Dimethyl Sulfoxide

One growth control well and one drug control well was also put in parallel for each isolate.

INCUBATION:

All microtitre plates were incubated at 35^{o}C and examined after 46-50 hours for *Candida sp* and after 70-72 hours for *C.neoformans.*

INTERPRETATION:

Minimum inhibitory concentration is the lowest concentration of an antifungal that causes specified reduction in visible growth in antifungal (broth dilution) susceptibility test. The magnitude of reduction in visible growth is assessed using the following numerical score for each microdilution well:

Score 4- No reduction in visible growth

Score 3- Slight reduction in visible growth

Score 2-Prominent reduction in visible growth (~50% of growth control)

Score 1- Slightly hazy

Score 0 -Optically clear or absence of visible growth

- MIC for Amphotericin B is read at the well which gives a 0 score
- MIC for Azoles is read at the well which gives a score of 2

ATCC *C.krusei* 6258 (QC strain) and ATCC *C.parapsilosis* 90018 (reference strain) were also tested in parallel for determination of MIC. Recommended 48 hours MIC limits for ATCC *C.krusei* are 1 - 4µg/ml for amphotericin B, 16 – 128µg/ml for fluconazole and 0.12 – 1µg/ml for voriconazole. For ATCC *C.parapsilosis*, the MIC limits are 0.5 – 4µg/ml for amphotericin B, 1 – 4µg/ml for fluconazole and 0.03 – 0.25µg/ml for voriconazole. Further, ATCC *C. neoformans* 32045 was also tested in addition to the above two control strains.

The MIC of antifungal agent was interpreted according to the values given in the table below[20]

Antifungal agent	**Susceptible (S)**	**Susceptible Dose Dependent (S-DD)**	**Resistant (R)**
Fluconazole	≤ 8 µg/ml	16-32 µg/ml	≥64 µg/ml
Voriconazole	≤ 1 µg/ml	2 µg/ml	≥4 µg/ml

For amphotericin B, the isolate is likely to be resistant if an MIC of ≥1 µg/ml is obtained

EPSILOMETER (E) TEST:

E test was performed for determining MIC of amphotericin- B and fluconazole to cryptococcal isolates. E test strips of both the drugs were obtained from Hi Media, Mumbai. Disposable plastic plates of 90mm containing RPMI 1640 and 2% glucose agar to a depth of 4mm were used. All the cryptococcal isolates and ATCC *C.neoformans* were subcultured on SDA for 48 hours and a yeast suspension equivalent to turbidity of 1 McFarland was prepared. A lawn culture of the suspension was made with a sterile swab and E strip was placed on the surface of the agar. The concentration gradient varied from 0.002- 32µg/ml for amphotericin- B and 0.016-256 µg/ml for fluconazole. The plates were incubated at 35^0C for 72 hours. MIC is the point of intersection of the growth inhibition zone with the strip. For amphotericicn B, the MIC was read at the point of complete inhibition (100%) of growth while for fluconazole, MIC was read at the point of approximately 80% inhibition of growth.

For comparing the performance of E test with that of the reference microbroth dilution test, Essential agreement (EA) and Categorical agreement (CA) were calculated[50]. A testing system is said to have "essential agreement" when the method under evaluation has an MIC within ± one 2-fold dilution compared with the reference method for MIC determination. "Categorical agreement" is when the interpretive results (susceptible, intermediate, or resistant) between a new method under evaluation and a standard reference method are the same. It is possible for there to be essential agreement with categorical disagreement. When this occurs, it is considered a "minor error" and frequently results in an intermediate susceptibility with one method and susceptible or resistant with another method. For drugs without an intermediate category, there cannot be minor errors. A "major error" occurs when the testing method determines that the organism is resistant, but the reference method determines that the organism is susceptible. Finally, a "very

major error" occurs when the converse is true—the testing method indicates the organism is susceptible but the reference method indicates that the organism is resistant.

DETERMINATION OF CD4 COUNT- CD4 count of the patients was determined using flow cytometer (Cyflow- Partec)

STATISTICAL ANALYSIS- Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 20.0. The proportional data of this study were tested using Pearson's Chi square analysis test χ^2

Results

RESULTS

A total of 175 CSF samples were collected during the study period from HIV positive patients presenting with features of meningitis.

TABLE 1- AGE AND SEX DISTRIBUTION OF HIV PATIENTS PRESENTING WITH FEATURES OF MENINGITIS (n=175)

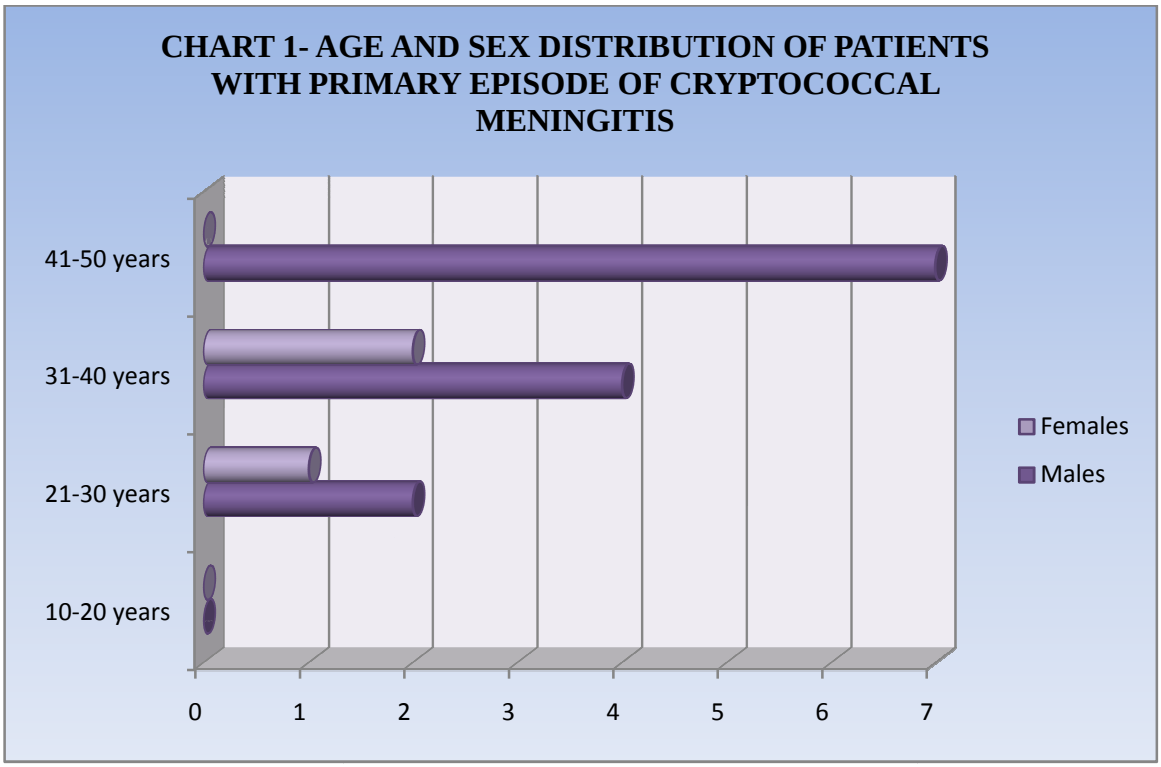
Age (years)	Males (n)	Females (n)
10-20	2	1
21-30	40	18
31-40	57	14
41-50	38	-
51-60	4	-
61-70	1	-
	142	**33**

The age of the patients varied from 14 to 65 years with a mean age of 38 yrs. Only 33 (18.86%) of the 175 patients were females.

Among these HIV patients, 19 patients were diagnosed with cryptococcal meningitis (CM), of which, 16 were primary episodes and 3 were symptomatic relapse episodes.

TABLE 2- AGE AND SEX DISTRIBUTION OF PATIENTS WITH CRYPTOCOCCAL MENINGITIS (n=19)

Age (years)	Primary episode (n=16)		Relapse (n=3)	
	Males	Females	Males	Females
20-30	2	1	1	-
31-40	4	2	1	-
41-50	7	-	1	-



Among the 16 patients who presented with a primary episode of cryptococcal meningitis, only 3 were females (18.75%). None of the female patients had a relapse episode during the study period.

TABLE 3- DISTRIBUTION OF SIGNS AND SYMPTOMS IN CRYPTOCOCCAL MENINGITIS (n=19)

Symptom/ Sign	Primary episode [n= 16] (%)	Relapse [n=3] (%)	Total [n=19] (%)
Headache	14 (87.5)	3 (100)	17 (89.47)
Vomiting	9 (56.25)	3 (100)	12 (63.16)
Fever	5 (31.25)	3 (100)	8 (42.11)
Altered sensorium	6 (37.5)	1 (33.33)	7 (36.84)
Neck rigidity	6 (37.5)	1 (33.33)	7 (36.84)
Visual disturbances	3 (18.75)	1 (33.33)	4 (21.05)
FND* - Paraparesis - Hemiparesis	3 (18.75) 2 (12.5) 1 (6.25)	-	3 (15.79) 2 (10.53) 1 (5.26)
Papilledema	1 (6.25)	1 (33.33)	2 (10.53)
Seizures (GTCS)	1 (6.25)	-	1 (5.26)
Skin lesions	-	1 (33.33)	1 (5.26)
Osteomyelitis	1 (6.25)	-	1 (5.26)
Autonomic dysfunction (urinary incontinence)	1 (6.25)	-	1 (5.26)

*FND- Focal neurological deficit

Headache was the predominant symptom observed in HIV patients presenting with symptoms of meningitis.

TABLE 4- TIME DURATION BETWEEN DIAGNOSIS OF HIV INFECTION AND FIRST EPISODE OF CRYPTOCOCCAL MENINGITIS (n= 16)

Diagnosis of HIV infection	No. of patients	Percentage (%)
On admission	3	18.75
<6 months	2	12.5
6- 12 months	1	6.25
13- 24 months	4	25
25-36 months	3	18.75
>36 months	3	18.75

Majority of the patients presented between 1-2 years of diagnosis of HIV infection. Cryptococcal meningitis was the AIDS defining illness in 3 patients.

CHART 2- DURATION BETWEEN DIAGNOSIS OF HIV INFECTION AND PRSENTATION WITH PRIMARY EPISODE OF CRYPTOCOCCAL MENINGITIS

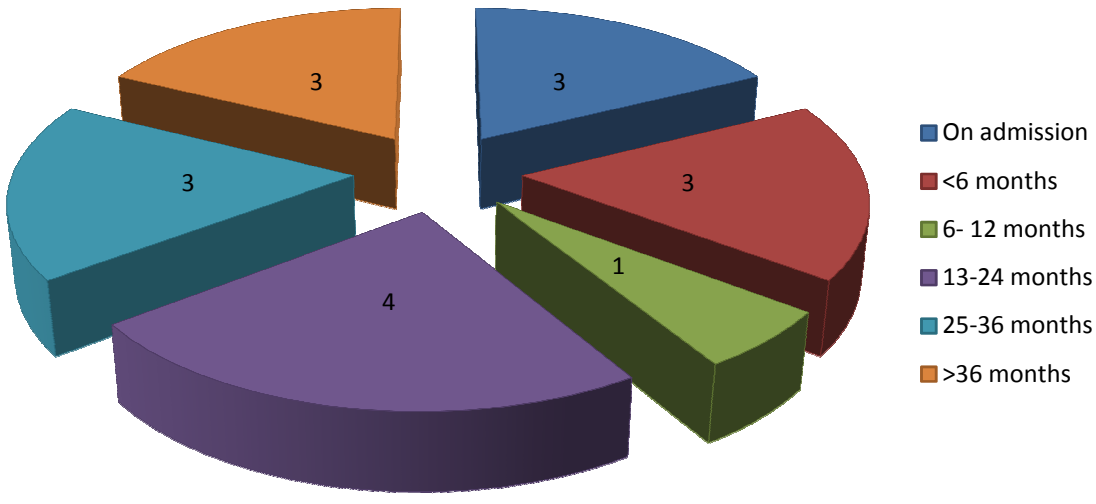


TABLE 5- CORRELATION BETWEEN CRYPTOCOCCAL MENINGITIS AND PRIOR TREATMENT WITH ANTI RETROVIRAL THERAPY (ART) AND/OR PROPHYLACTIC FLUCONAZOLE (n=19)

Cryptococcal meningitis (CM)		No.of patients on ART	No.of patients on ART and T. Fluconazole
First episode (n= 16)	(n=3)*	-	-
	(n=13)	9 (69.23%)	3 (23.07%)
Relapse (n=3)		2 (66.67%)	2 (66.67%)

*Patients diagnosed with HIV infection only after admission

More than half of the patients with prior diagnosis of HIV infection were receiving ART at the time of presentation with CM. In addition, 3 patients were also receiving T.fluconazole as a prophylactic measure against opportunistic infections. Only one of the 3 patients who presented with relapse had failed to take secondary fluconazole prophylaxis and was also not started on HAART.

CHART 3- CORRELATION BETWEEN CM AND PRIOR INTAKE OF ART OR FLUCONAZOLE

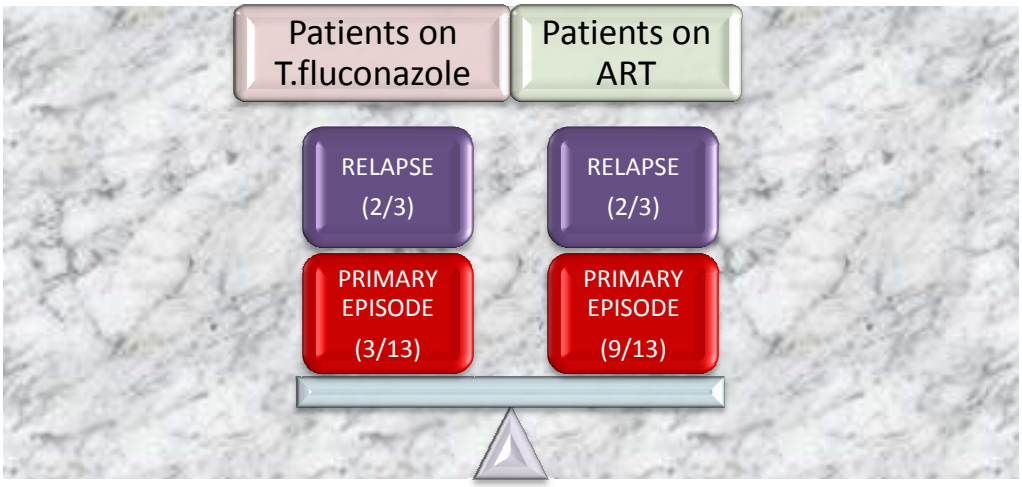


TABLE 6- BIOCHEMICAL ANALYSIS OF CSF IN CRYPTOCOCCAL MENINGITIS (n=19)

CSF	No. of Patients	Percentage
GLUCOSE		
▪ Reduced	6	31.57
▪ Normal	12	63.15
▪ Elevated	1	5.26
PROTEIN		
▪ Reduced	Nil	-
▪ Normal	5	26.31
▪ Elevated	14	73.68
Cell count*		
▪ Normal	2	10.52
▪ Elevated	15	78.94

*****complete absence of cells in the CSF (n=2)

Normal CSF glucose level in an adult is 40-70 mg/dl, protein is 15-40 mg/dl and cell count is <5 cells/µl. The CSF glucose level was normal in majority of the patients while protein and cell counts were elevated.

TABLE 7- COMPARISON OF DETECTION OF CRYPTOCOCCAL MENINGITIS BY VARIOUS TESTS (n=19)

Test	First episode (n=16)		Relapse (n=3)	
	Positive	**Negative**	**Positive**	**Negative**
India ink	11 (68.75%)	5 (31.25%)	2 (66.67%)	1 (33.33%)
Gram stain	7 (43.75%)	9 (56.25%)	1 (33.33%)	2 (66.67%)
Latex Agglutination Test (LAT)	16 (100%)	-	3 (100%)	-
Culture	12 (75%)	4 (25%)	1 (33.33%)	2 (66.67%)

LAT detected capsular polysaccharide in all patients with CM

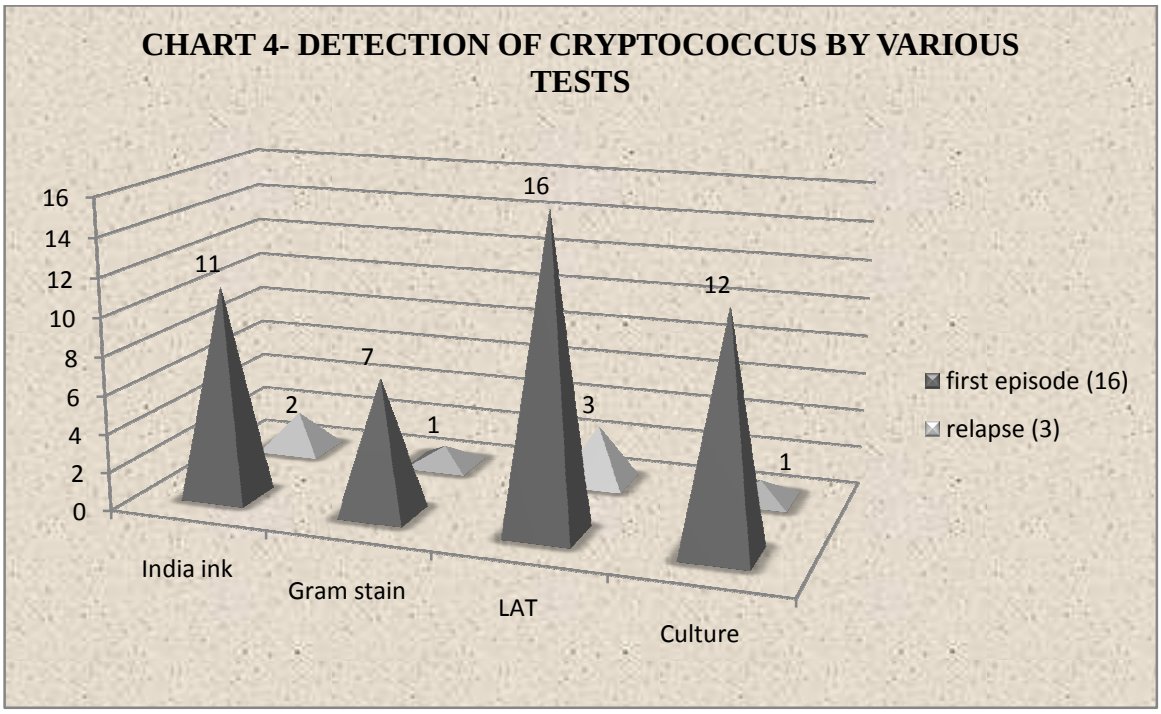
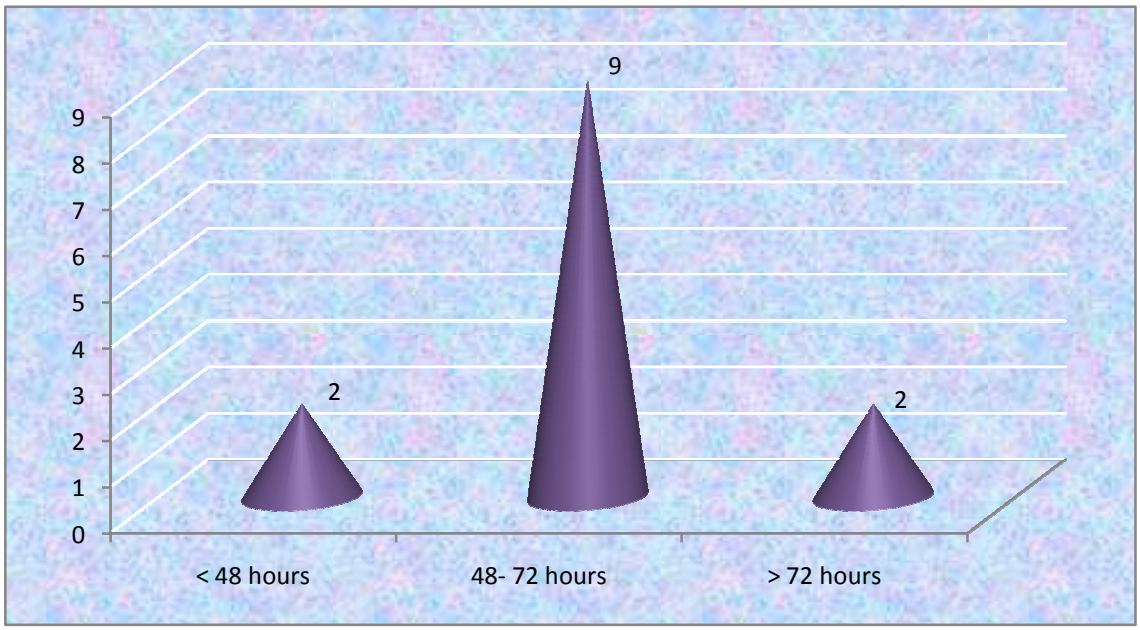


TABLE 8– DURATION OF GROWTH OF CRYPTOCOCCAL ISOLATES ON SDA (n=13)

Duration of growth on SDA	No. of isolates	Percentage
<48 hours	2	15.38
48- 72 hours	9	69.23
>72 hours	2	15.38
	13	**100**

CHART 5- DURATION OF GROWTH OF CRYPTOCOCCAL ISOLATES ON SDA

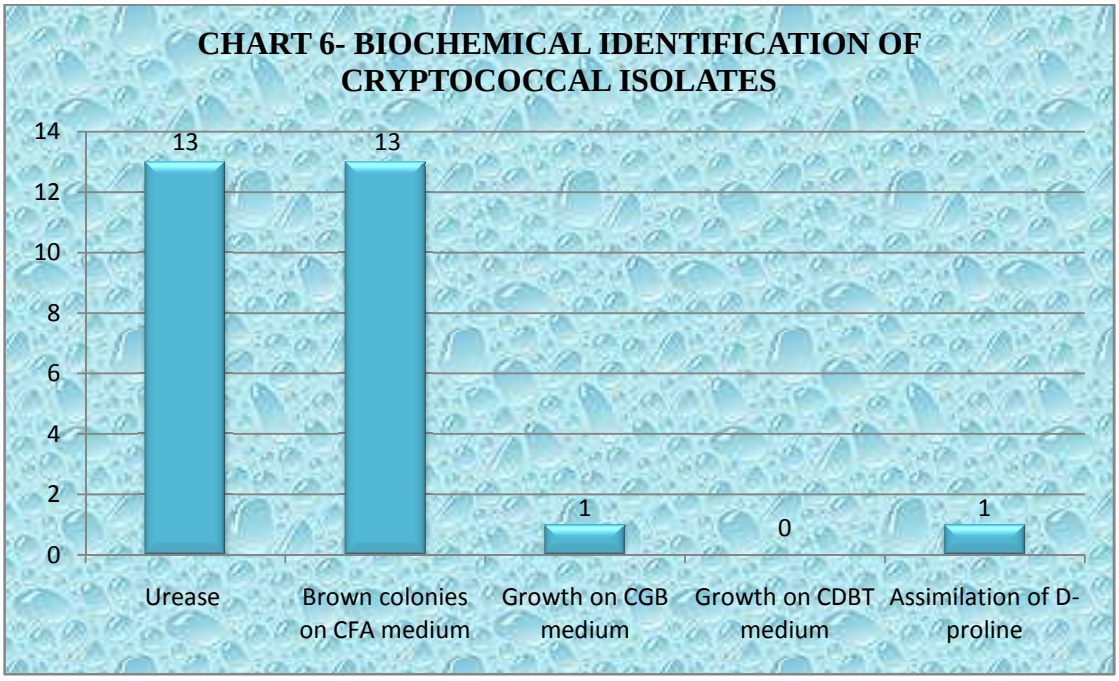


Majority of the cryptococcal isolates grew within 72 hours of inoculation of CSF on SDA. However, one of the isolate grew on the 5th day and another grew on the 7th day only.

TABLE 9- BIOCHEMICAL IDENTIFICATION OF CULTURE POSITIVE CRYPTOCOCCAL ISOLATES

Biochemical test	No. of isolates	
	Positive	Negative
Hydrolysis of urea (n=13)	13	13
Brown colonies on CFA medium (n=13)	13	13
Growth on CGB medium (n=13)	1	12
Assimilation of D-proline (n=13)	1	12
Growth on CDBT medium (n=12)	0	12

CFA- Caffeic acid ferric citrate agar, CGB- Canavanine Glycine Bromothymol blue medium, CDBT- Creatinine Dextrose Bromothymol blue Thymine medium



All the 13 cryptococcal isolates hydrolysed urea and formed brown colonies on CFA medium. But only 1 isolate grew on CGB medium and assimilated proline.

TABLE 10- ASSIMILATION OF CARBOHYDRATES BY CRYPTOCOCCAL ISOLATES (n=13)

Test isolates (13)		Inositol	Dextrose	Lactose	Maltose	Sucrose	Cellobiose
C.neoformans (12)	+	12	12	0	12	12	10
	-	0	0	12	0	0	2
C.gattii (1)	+	1	1	0	1	1	0
	-	0	0	1	0	0	1

All the *C.neoformans* isolates assimilated inositol, dextrose, maltose and sucrose. But 2 of them failed to assimilate cellobiose and none of the isolates assimilated lactose. The *C.gattii* isolate assimilated inositol, dextrose, maltose and sucrose but not lactose and cellobiose.

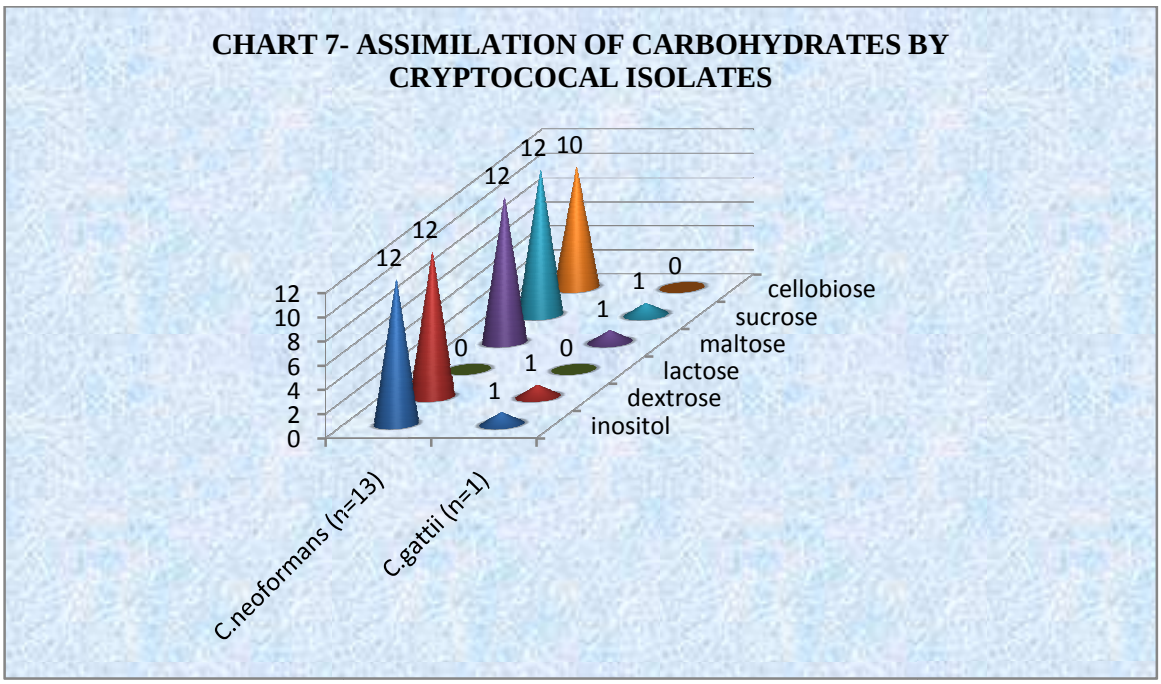
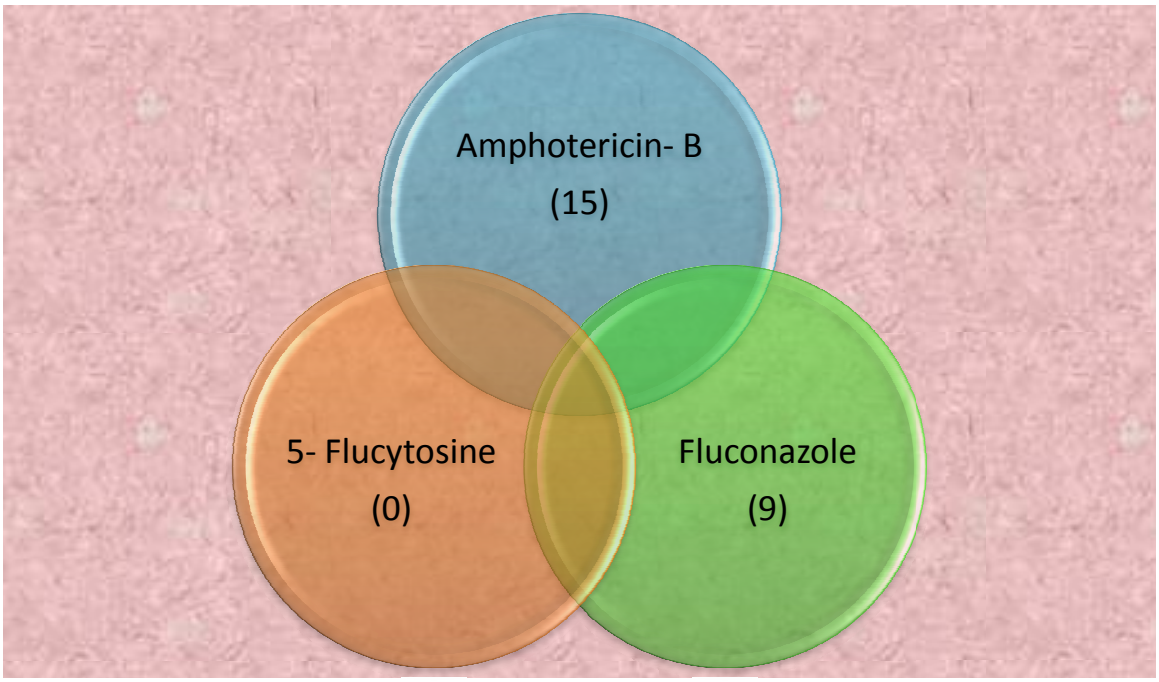


TABLE 11- TREATMENT RECEIVED BY PATIENTS FOR FIRST EPISODE OF CRYPTOCOCCAL MENINGITIS (n=16)

Treatment	No.of patients (n=16)	Outcome
Amphotericin-B + 5-Flucytosine	-	-
Amphotericin-B alone	6	2- survived, 4- died
Amphotericin-B + Fluconazole	9	7- survived, 2- died
Others	1	Died



None of the patients received 5-FC as part of primary therapy for Cryptococccal meningitis. 6 of the 16 patients died during the primary episode despite institution of amphotericin-B and 1 patient died even before starting amphotericin-B.

CHART 8- CD4 COUNT OF PATIENTS WITH CRYPTOCOCCAL MENINGITIS*

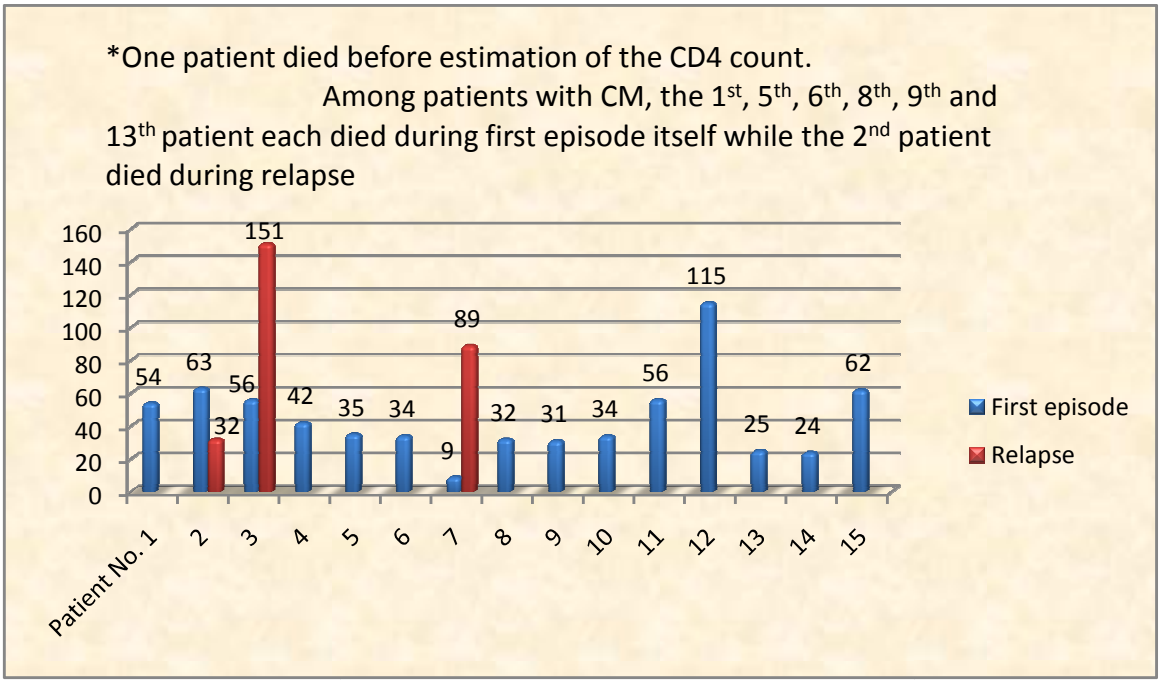


TABLE 12- CORRELATION OF CD4 COUNT WITH SURVIVAL OF THE PATIENTS WITH CRYPTOCOCCAL MENINGITIS (n=16)

CD4 count (cells/µl)	No. of patients (n=15 + 1*)	Survived	Died
<40	8	2	6
40-100	6	5	1
>100	1	1	-

*One patient died before estimation of the CD4 count

For patients with a relapse episode of CM, CD4 count of the relapse episode is shown in the above table. Majority of the patients who died had CD4 count <40cells/µl at the time of presentation with CM. Statistically significant association was found between death of the patient and a CD4 count <40cells/µl (p= 0.018)

TABLE 13- MIC OF AMPHOTERICIN B FOR CRYPTOCOCCAL ISOLATES BY MICROBROTH DILUTION METHOD (n=13)

Test isolates (13)	MIC (µg/ml)									
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
C.neoformans (12)	-	-	2	9	1	-	-	-	-	-
C.gattii (1)	-	-	-	-	1	-	-	-	-	-

There are no definitive susceptible CLSI breakpoints for amphotericin-B to cryptococcal strains. However, any isolate with MIC >1µg/ml is considered to be resistant to amphotericin-B. The MIC of amphotericin-B varied from 0.12-0.5 µg/ml.

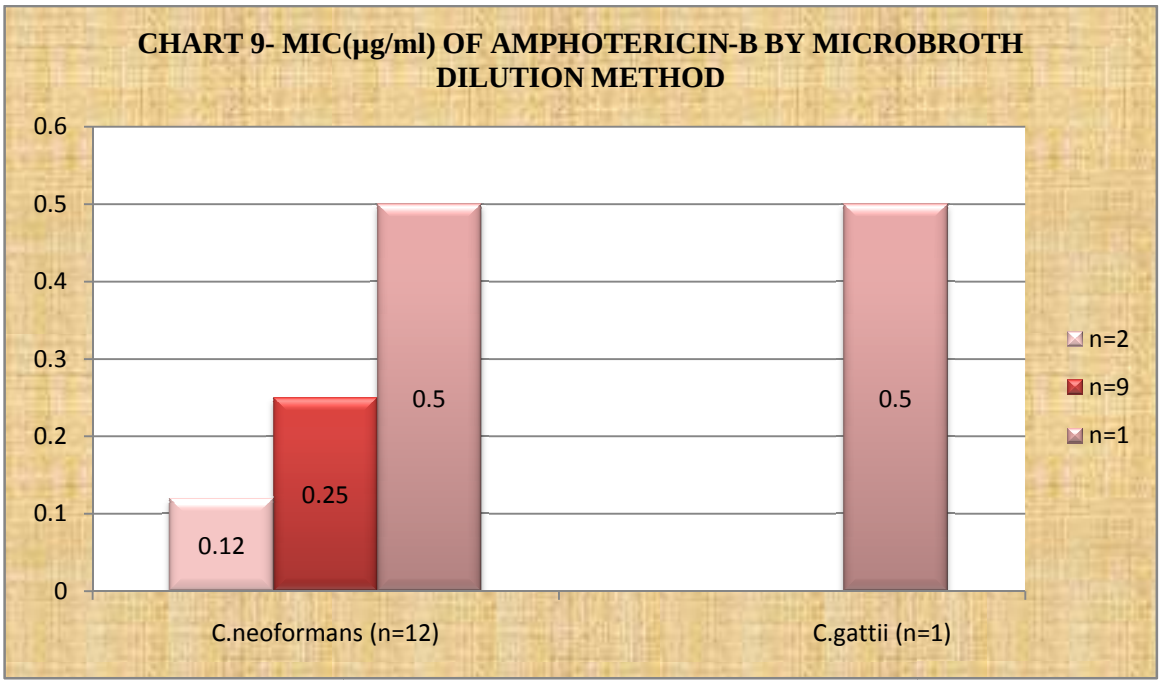


TABLE 14- MIC OF AMPHOTERICIN B FOR CRYPTOCOCCAL ISOLATES BY EPSILOMETER TEST (n=13)

Test isolates (13)	MIC (µg/ml)									
	0.002	0.004	0.008	0.016	0.032	0.064	0.128	0.256	0.5	1
C.neoformans (12)	-	-	-	2	3	2	4	1	-	-
C.gattii (1)	-	-	-	-	-	-	1	-	-	-

MIC of amphotericin-B by E test varied from 0.016-0.256 µg/ml.

TABLE 15- COMPARISON OF MIC LEVELS OF AMPHOTERICIN-B OBTAINED BY MICROBROTH DILUTION METHOD AND E TEST (n=13)

MIC (µg/ml)									
Microbroth dilution method		0.008	0.016	0.032	0.064	0.128	0.256	0.5	1
	C.neoformans (12)	-	-	-	-	2	9	1	-
	C.gattii (1)	-	-	-	-	-	-	1	-
E test	*C.neoformans* (12)	-	2	3	2	4	1	-	-
	C.gattii (1)	-	-	-	-	-	-	1	-

Isolates of *C.neoformans* were found to have a relatively lower MIC for amphotericin-B when tested by E test in comparison with the microbroth dilution method. The essential agreement [EA] (MIC of E test within $\pm$ one 2 fold dilution when compared with the reference microbroth dilution method) is 61.5% as 8 of the 13 cryptococcal isolates had MIC ranging from 0.064- 0.5 µg/ml which is within one 2 fold dilution of the

range obtained by the reference method (0.12-0.5 µg/ml). The categorical agreement [CA] (interpretive results: S, I, R are same between E test and reference microbroth dilution method) is 100% as all the cryptococcal isolates were susceptible to amphotericin-B by both microbroth dilution method and E test.

Significant association was found between testing cryptococcal isolates with E test and obtaining lower MIC values ≤0.012 µg/ml for amphotericin-B compared to microbroth dilution method (p= 0.002)

TABLE 16- MIC OF FLUCONAZOLE FOR CRYPTOCOCCAL ISOLATES BY MICROBROTH DILUTION METHOD (n=13)

Test isolates (13)	**MIC (µg/ml)**									
	0.12	0.25	0.5	1	2	4	8	16	32	64
C.neoformans (12)	-	-	5	6	1	-	-	-	-	-
C.gattii (1)	-	-	-	-	1	-	-	-	-	-

According to CLSI M27-A3, cryptococcal isolates tested by microbroth dilution method and exhibiting MIC ≤8 µg/ml are susceptible, 16-32 µg/ml are susceptible- dose dependant and ≥64 µg/ml are resistant to fluconazole respectively. The MIC of fluconazole varied from 0.5-2 µg/ml.

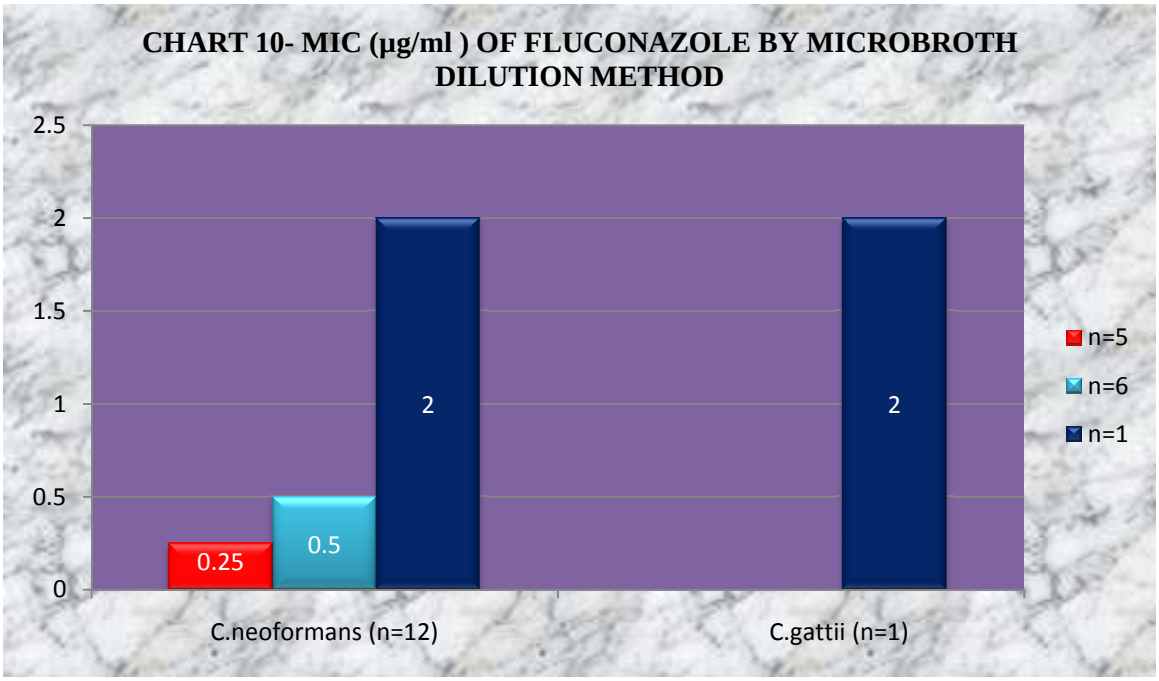


TABLE 17- MIC OF FLUCONAZOLE FOR CRYPTOCOCCAL ISOLATES BY EPSILOMETER TEST (n=13)

Test isolates (13)	MIC (µg/ml)									
	0.16	0.32	0.64	0.128	0.256	0.5	1	2	4	8
C.neoformans (12)	-	-	-	-	-	-	3	3	4	2
C.gattii (1)	-	-	-	-	-	-	-	-	-	1

MIC of fluconazole by E test varied from 1-8 µg/ml

TABLE 18- COMPARISON OF MIC LEVELS OF FLUCONAZOLE OBTAINED BY MICROBROTH DILUTION METHOD AND E TEST (n=13)

MIC (µg/ml)									
Microbroth dilution method		0.128	0.256	0.5	1	2	4	8	16
	C.neoformans (12)	-	-	5	6	1	-	-	-
	C.gattii (1)	-	-	-	-	1	-	-	-
E test	*C.neoformans* (12)	-	-	-	3	3	4	2	-
	C.gattii (1)	-	-	-	-	-	-	1	-

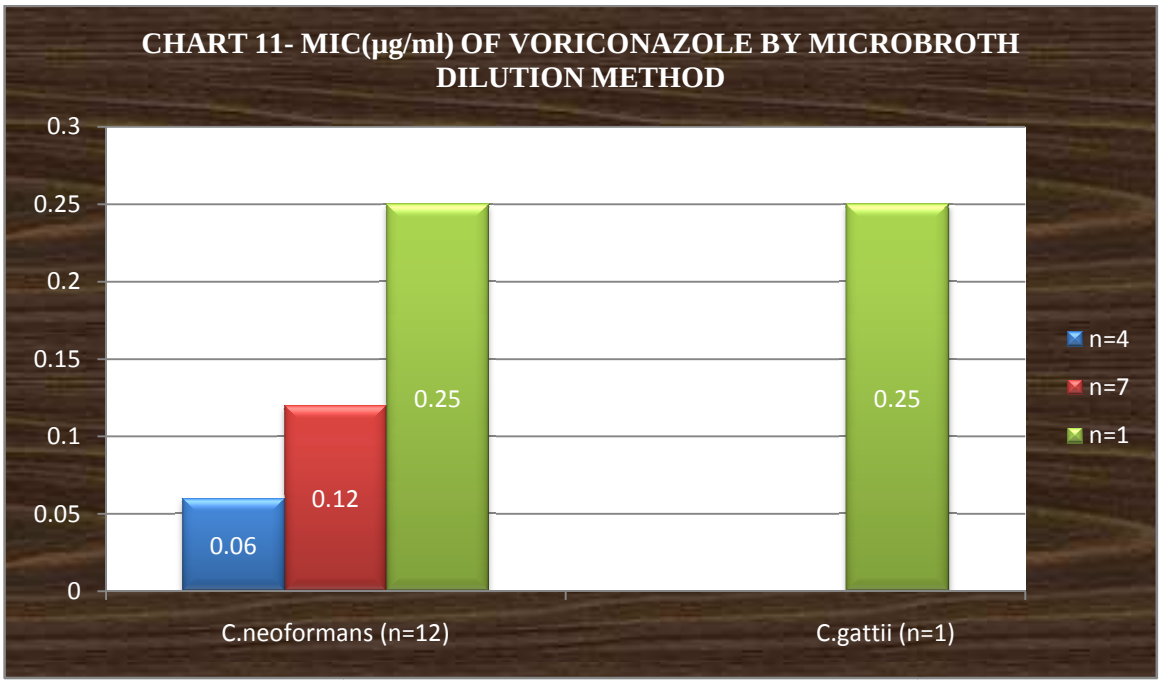
Isolates of both *C.neoformans* and *C.gattii* were found to have a relatively higher MIC for fluconazole when tested by E test in comparison with the microbroth dilution method. The essential agreement [EA] is 76.9% as 10 of the 13 cryptococcal isolates had MIC ranging from 1- 4µg/ml which is within one 2 fold dilution of the range obtained by the reference method (0.5-2 µg/ml). The categorical agreement [CA] is 100% as all the cryptococcal isolates were susceptible to fluconazole by both microbroth dilution method and E test.

Significant association was observed between testing cryptococcal isolates by E test and obtaining higher MIC values ≥4µg/ml for fluconazole compared to microbroth dilution method (p=0.008).

TABLE 19- MIC OF VORICONAZOLE FOR CRYPTOCOCCAL ISOLATES BY MICROBROTH DILUTION METHOD (n=13)

Test isolates (13)	MIC (µg/ml)									
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
C.neoformans (12)	-	4	7	1	-	-	-	-	-	-
C.gattii (1)	-	-	-	1	-	-	-	-	-	-

According to CLSI M27-A3, cryptococcal isolates tested by microbroth dilution method and exhibiting MIC ≤1 µg/ml are susceptible, 2 µg/ml are susceptible- dose dependant and ≥4 µg/ml are resistant to voriconazole respectively. The MIC of voriconazole varied from 0.06-0.25µg/ml.



All the cryptococcal isolates were sensitive to voriconazole by microbroth dilution method with MIC <1µg/ml.

TABLE 20- SUSCEPTIBILITY PATTERN OF CRYPTOCOCCAL ISOLATES TO ANTIFUNGAL AGENTS (n=13)

Antifungal drug	Microbroth dilution method (Susceptible isolates in %)	E test (Susceptible isolates in %)
Amphotericin- B	100	100
Fluconazole	100	100
Voriconazole	100	Not tested

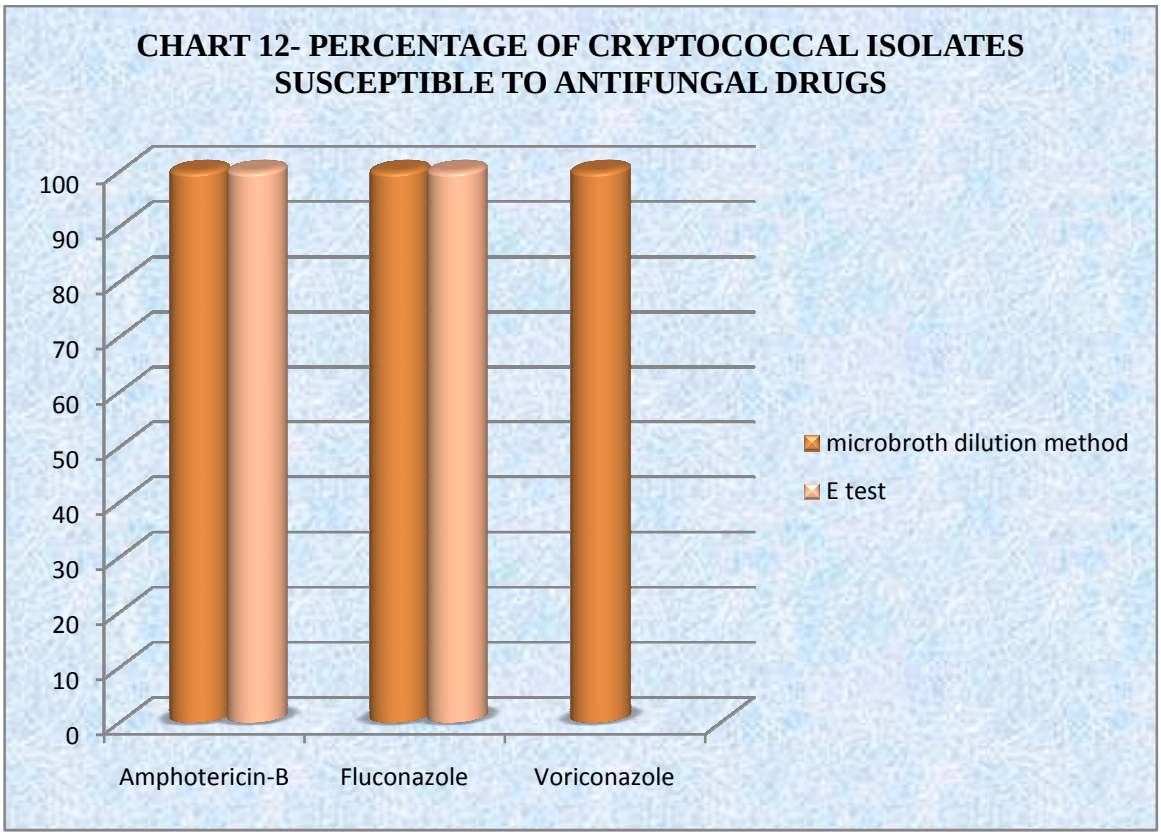


FIGURE 1- SWELLING OF LEFT HAND IN A PATIENT WITH OSTEOMYELITIS OF FIRST METACARPAL BONE

FIGURE 2- MRI SCAN OF THE PATIENT SHOWING PRESENCE OF HYPODENSE LESION AT THE BASE OF LEFT FIRST METACARPAL BONE

FIGURE 3- CUTANEOUS LESIONS IN A PATIENT WITH DISSEMINATED CRYPTOCOCCAL INFECTION

FIGURE 4- CT SCAN SHOWING DILATED VENTRICLES IN A PATIENT WITH OBSTRUCTIVE HYDROCEPHALUS SECONDARY TO CHRONIC CM

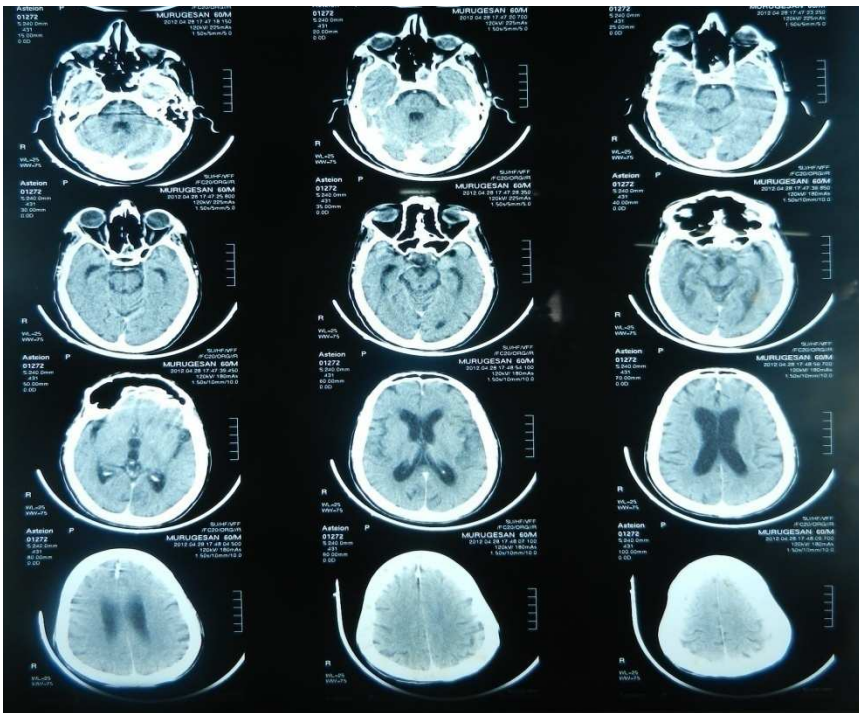


FIGURE 5- INDIA INK PREPARATION SHOWING PRESENCE OF CAPSULATED BUDDING YEAST CELLS IN THE CSF

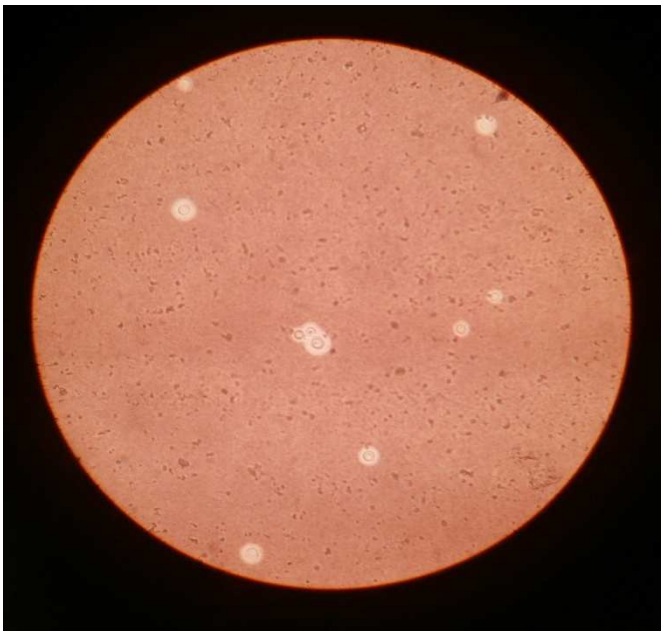


FIGURE 6- MODIFIED INDIA INK PREPARATION WITH 2% MERCUROCHROME SHOWING THE 3 LAYERS OF THE CAPSULE OF CRYPTOCOCCUS

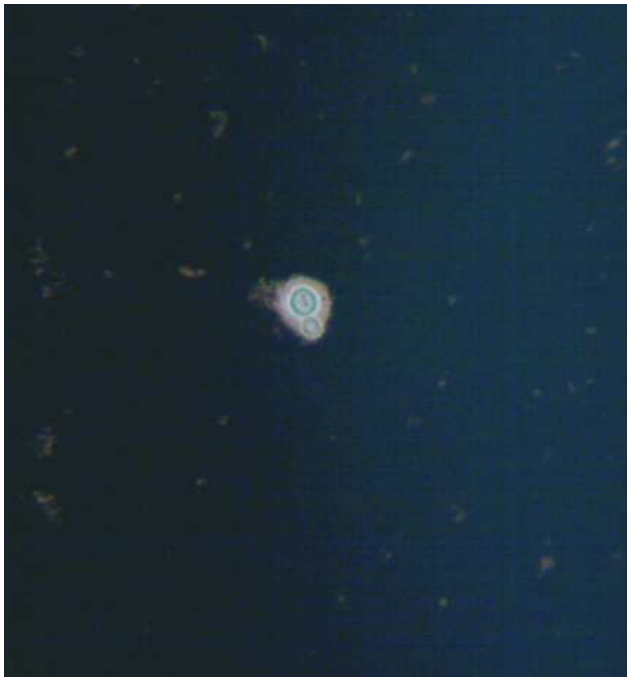


FIGURE 7- GRAM POSITIVE CAPSULATED BUDDING YEAST CELLS IN THE CSF

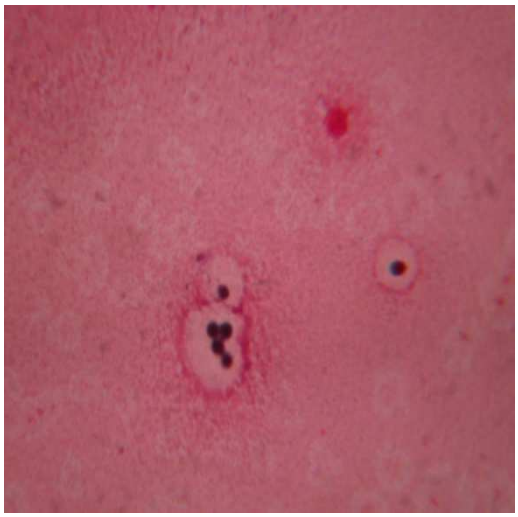


FIGURE 8- LATEX AGGLUTINATION TEST POSITIVE FOR CRYPTOCOCCAL POLYSACCHARIDE IN THE CSF

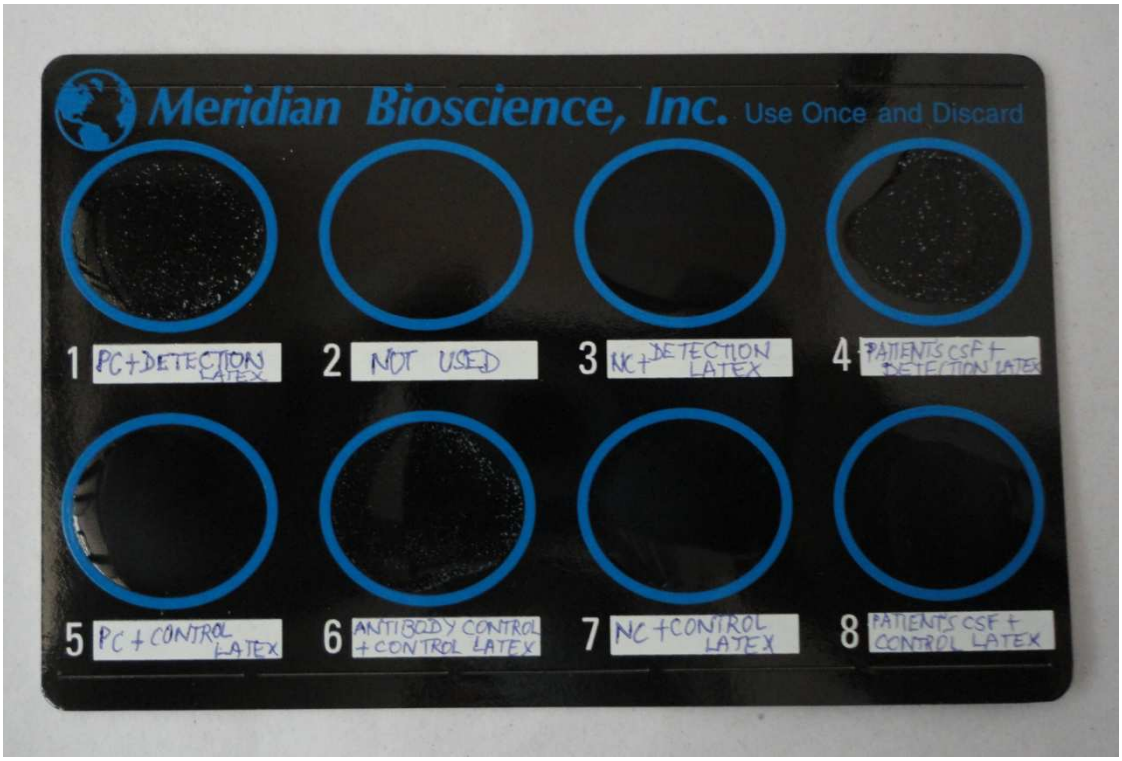


FIGURE 9- SDA SLOPES SHOWING GROWTH OF CRYPTOCOCCUS FROM CSF

FIGURE 10- SDA PLATE WITH CRYPTOCOCCUS ISOLATED FROM SKIN LESION

FIGURE 11- CONTROL STRAINS USED FOR BIOCHEMICAL REACTIONS

FIGURE 12- TEST ISOLATE SHOWING A POSITIVE UREASE REACTION

FIGURE 13- GROWTH OF CRYPTOCOCCUS ON CAFFEIC ACID MEDIUM

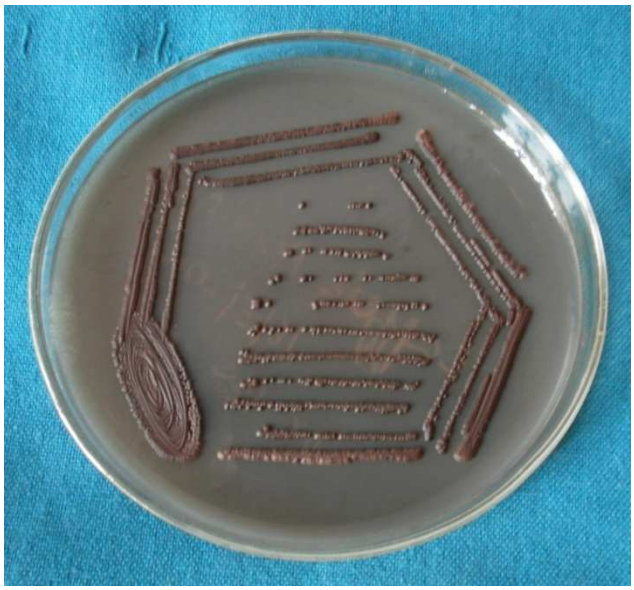


FIGURE 14- CFA MEDIUM SHOWING BROWN COLONIES OF CRYPTOCOCCUS NEOFORMANS AND WHITE COLONIES OF CANDIDA ALBICANS

Left- ATCC *C.neoformans* (Positive control), Bottom- ATCC *C.albicans* (Negative control), Top- Test isolate

FIGURE 15- GROWTH OF CRYPTOCOCCUS ON CGB MEDIUM

Left- C.gattii (positive control), Right- ATCC C.neoformans (negative control),

Middle- Test isolate

FIGURE 16- CGB AGAR PLATE WITH GROWTH OF C.GATTII ISOLATE

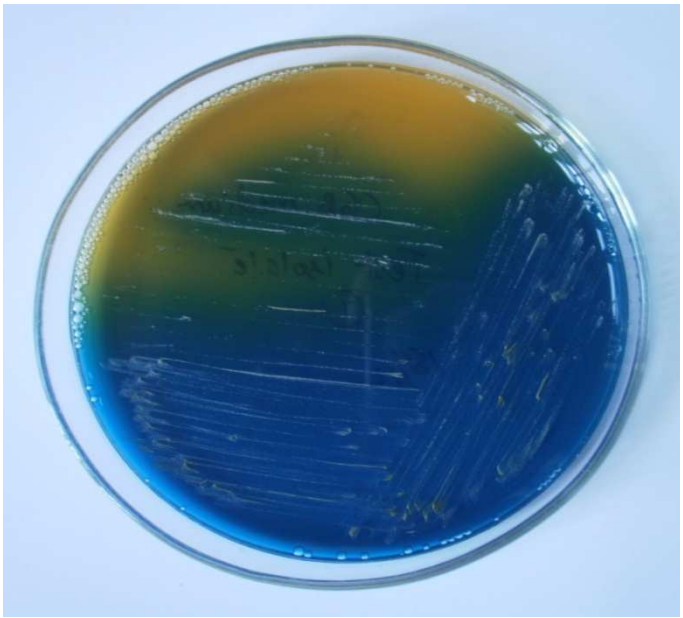


FIGURE 17- CDBT MEDIUM USED FOR VARIETY DIFFERENTIATION OF CRYPTOCOCCUS NEOFORMANS

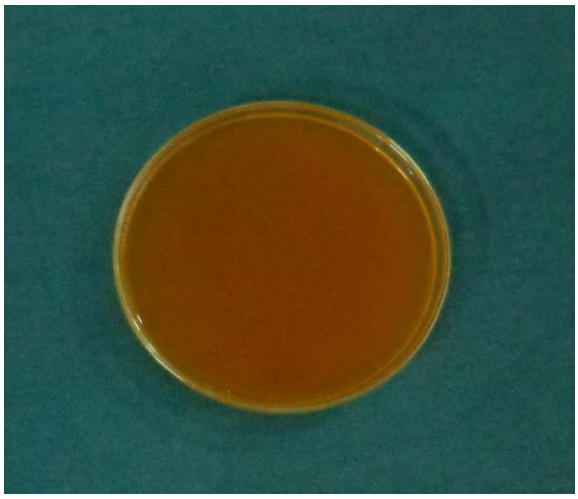


FIGURE 18- AUXANOGRAPHIC TECHNIQUE FOR ASSIMILATION OF CARBOHYDRATES BY CRYPTOCOCCUS NEOFORMANS

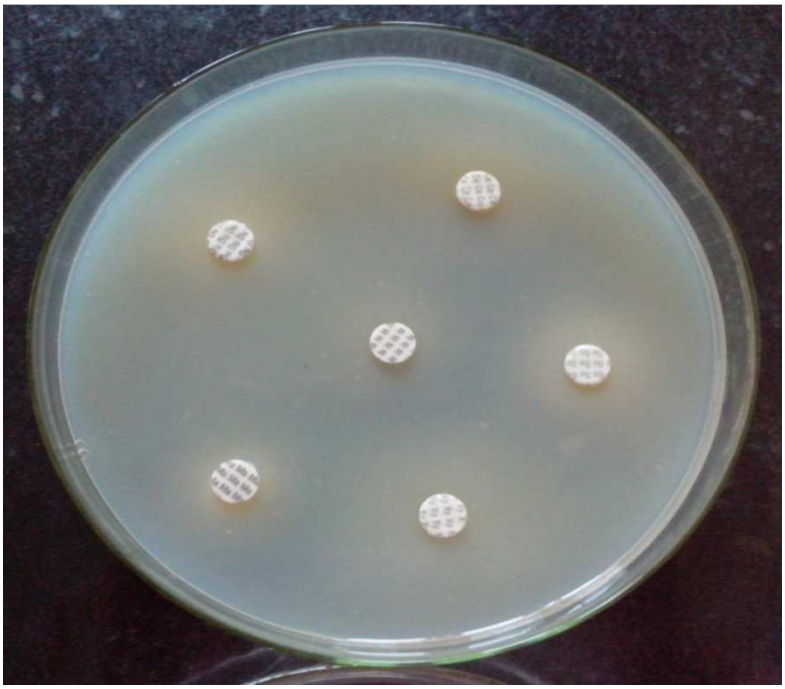


FIGURE 19- ABSENCE OF ASSIMILATION OF PROLINE BY TEST ISOLATE OF CRYPTOCOCCUS NEOFORMANS

FIGURE 20- ASSIMILATION OF PROLINE BY TEST ISOLATE OF CRYPTOCOCCUS GATTII

FIGURE 21- DETERMINATION OF AMPHOTERICIN- B MIC BY MICROBROTH DILUTION METHOD

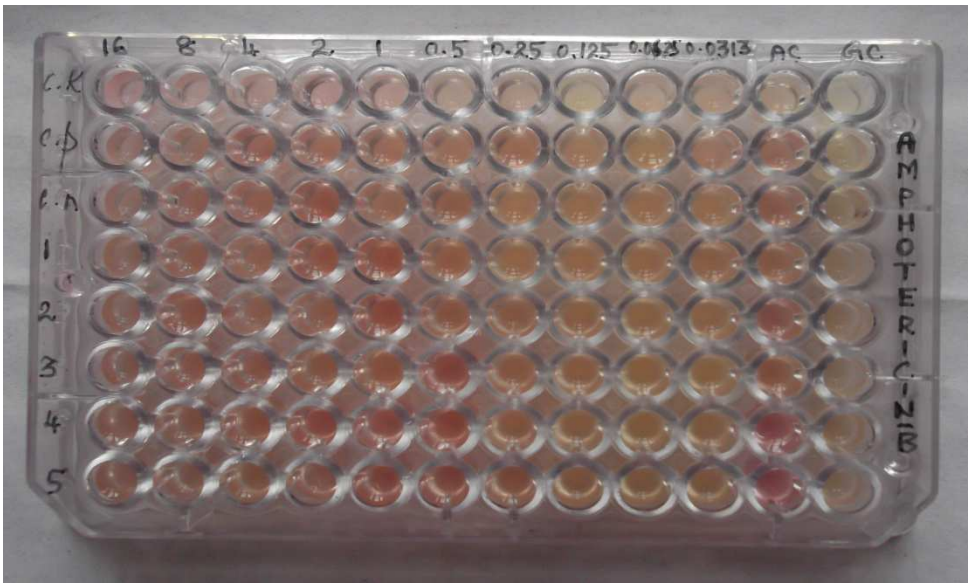


FIGURE 22- MIC DETERMINATION OF AMPHOTERICIN- B USING E TEST

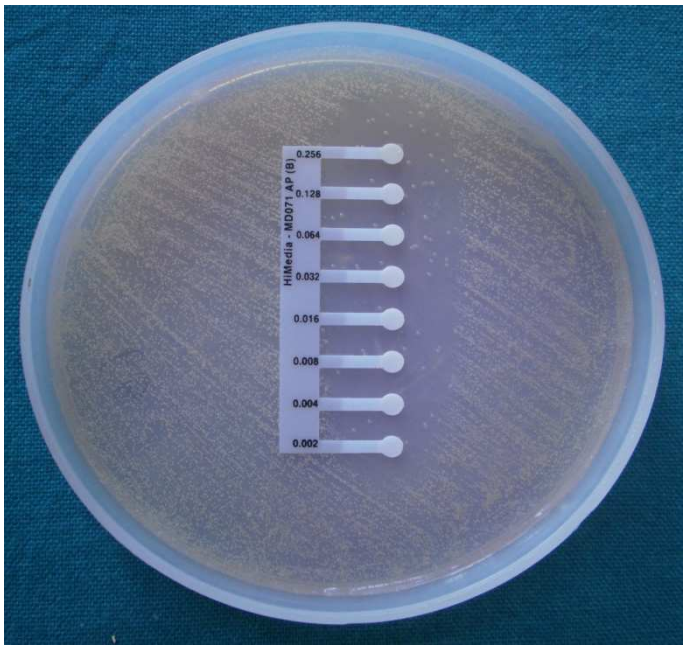


FIGURE 23- DETERMINATION OF FLUCONAZOLE MIC BY MICROBROTH DILUTION METHOD

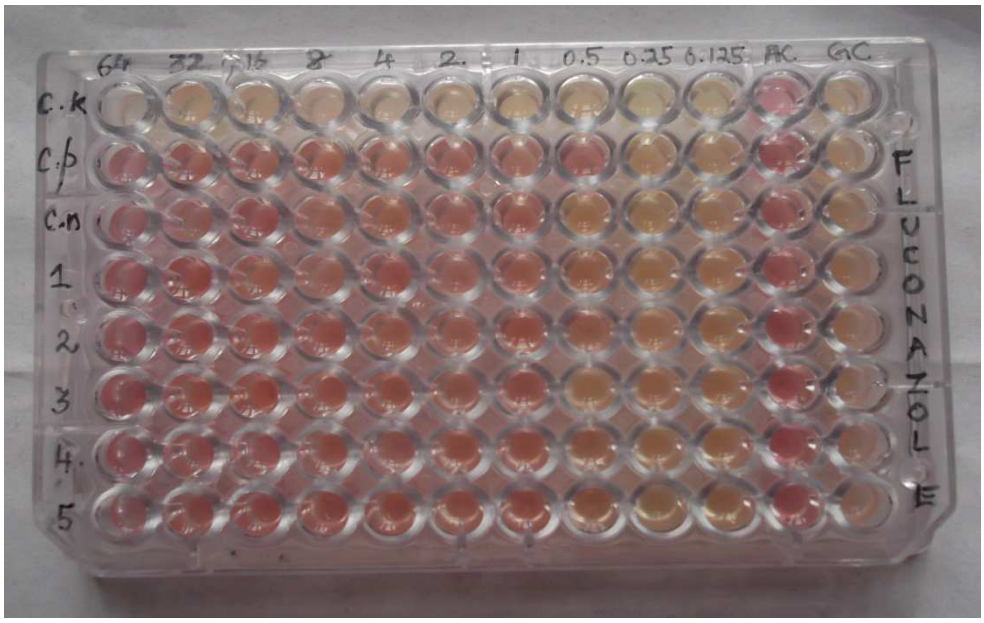


FIGURE 24- MIC DETERMINATION OF FLUCONAZOLE BY E TEST

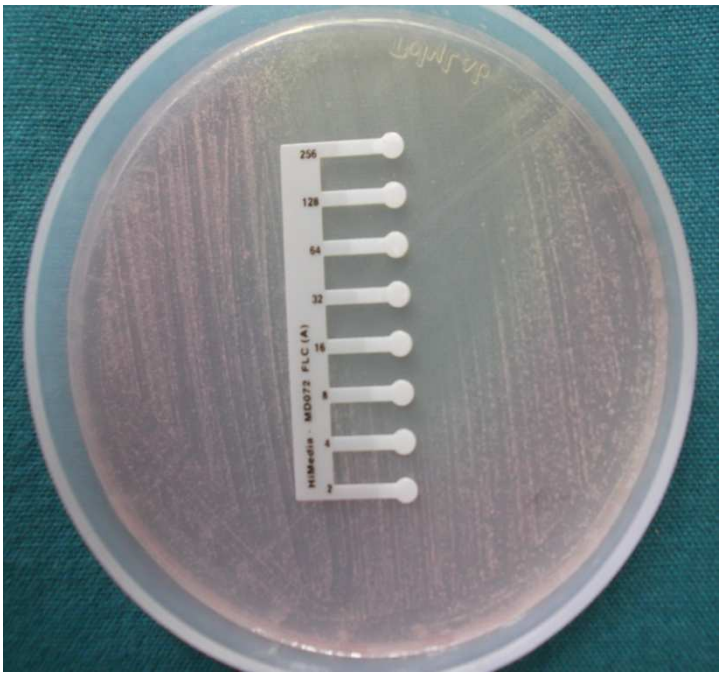
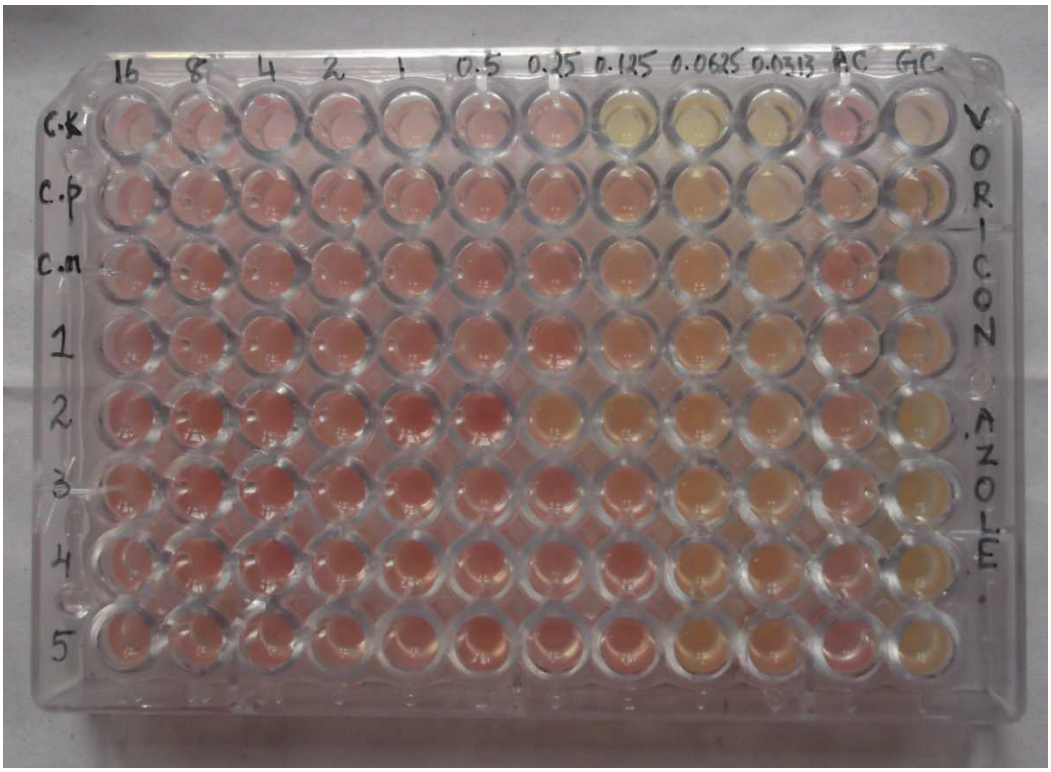


FIGURE 25- DETERMINATION OF VORICONAZOLE MIC BY MICROBROTH DILUTION METHOD



Discussion

DISCUSSION

Before the advent of the AIDS pandemic, relatively few cases of infections due to *C.neoformans* were reported and the rise in incidence of cryptococcosis paralleled the rise in HIV infection[19]. In a study carried out at PGIMER- Chandigarh, the annual incidence of CM increased 15 fold in 1995-99 when compared to 1970-82 synchronous with the spread of HIV cases in India[15]. Another study carried out at AIIMS, New Delhi (1992-2004) showed that HIV- *Cryptococcus* co-infection rose from 20% in 1992 to 49% in 2004. However, with early recognition of HIV infection and widespread access to ART, the incidence of cryptococcosis is now on the decline. In a report from France, the incidence of CM has declined by 46% in 1997-2001 when compared to 1985-1996 [21]. In India too, due to the active intervention by NACO and implementation of NACP, there has been an appreciable fall in the incidence of not only CM but all other opportunistic infections as well in patients with AIDS[70]. The prevalence of CM in HIV reactive patients was found to be 10.86% in this study. It is lower when compared to 12.9% in a study by Nigam *et al* conducted between March 2009 and February 2012 at BHU, Varanasi[70].

Majority of patients in this study were males (81.25%) similar to other studies in India and elsewhere (Table 2). Male to female ratio of 5.3:1 was observed. Cryptococcal meningitis is more frequently observed in men than women[42, 44, 81] and may reflect differences in exposure rather than susceptibility[76]. Most of the patients affected with CM in this study were in the age group of 41-50 years (43.75%) which is higher when compared to other studies. In a study by Thakur *et al*, 53% of patients were <40 years and only 15% were in the age group of 40-50 years[92].

Headache lasting more than a week was the predominant symptom (89.47%) in most of the patients in this study (Table 3). This is comparable to a study by Chakrabarti *et*

al where headache was seen in 90% of patients[49] and another study by Lakshmi *et al* where 92.31% of patients presented with headache[55]. Vomiting was noticed in 63.16% of patients while fever was observed in 42.11% of patients. Altered sensorium and/or terminal neck stiffness was seen in 36.84% of patients. However, Chakrabarti *et al* [49] and Lakshmi *et al* [55]had found altered sensorium in 45% and 71.79% of their patients respectively. Visual disturbances, predominantly blurring was noticed in 21.05% of patients in this study. Thakur *et al* had found diplopia in 16.7% of patients in their study[92]. Papilledema was observed in 10.53% of patients which is comparable to 9% found in a study by Aslam *et al* [5]. Motor deficits (para/hemiparesis) were seen in 15.79% of patients. In this study, seizures were noticed in only one patient who presented with 2 episodes of GTCS. Autonomic disturbance in the form of urinary incontinence was found in one of the patients. Previous reports too have described the occurrence of myelitis- like syndrome in cryptococcal meningitis[34]. Findings of CT scans were non- specific except for presence of obstructive hydrocephalus in one patient. In addition to the above features of meningitis, extrameningeal symptoms were found in two patients in this study. One patient presented with 10 days duration of cutaneous manifestations in the face, chest and back. On examination, several papulovesicular lesions were seen in the involved areas of the skin. It has been reported that involvement of the skin is a marker of disseminated cryptococcal infection until proved otherwise. Varied skin manifestations can occur in cryptococcosis including macules, papules, plaques, nodules, vesicles or ulcers[22]. Culture of swabs taken from the base of the vesicle in this patient yielded growth of yeast like colonies on SDA in 72 hours. The other patient with disseminated infection presented with a vague, painful swelling in the left hand of 2 months duration. MRI showed a hypodense cortical lesion at the base of the 1st metacarpal surrounded by hypodense areas in the soft tissue of the left hand. After treating the patient for CM, a sequestrectomy of the involved bone was

performed with clinical diagnosis of osteomyelitis which also grew yeast like colonies on SDA. The yeast like isolates obtained from skin and bone of the two patients were identified as *C.neoformans* based on hydrolysis of urea and production of brown colonies on CFA.

The duration between diagnosis of HIV infection and occurrence of primary episode of cryptococcal meningitis was less than 2 years in 7 of the 16 patients (Table 4). In 3 patients, a diagnosis of HIV infection was made only after admission with symptoms of meningitis. So, in this study CM was the AIDS defining illness in 18.75% of patients. Earlier studies showed a higher rate and in a study by Chakrabarti *et al* CM was the AIDS defining illness in 75% of their patients[49]. The fact that only 3 patients in this study were unaware of their HIV status reflects the trend of earlier diagnosis of HIV infection in the community.

Among the 13 patients in whom a diagnosis of HIV infection was made prior to the presentation with CM, 9 (69.23%) were on ART (Table 5). The duration of prior treatment with ART by the patients varied from 1 month to 2 years. Moreover, 3 (23.07%) patients were also on T. fluconazole in addition to ART as a prophylactic measure against opportunistic infections. Despite the intake of these drugs, the patients developed CM probably owing to their low CD4 counts (<100/µl) which failed to contain the infection. In a study by Baradkar *et al* [6] conducted between 2006 and 2007, only 21.05% of patients with CM were on prior ART. These findings reiterate that there is better availability and wider access to ART among HIV reactive cohorts.

Biochemical analysis of CSF showed normal glucose levels in majority (63.15%) of the patients (Table 6). However, protein and cell counts were found to be elevated in 73.68% and 78.94% of patients respectively in this study. In patients with raised cell

counts, the predominant cells were lymphocytes. No cells could be detected in the CSF of 2 (10.5%) patients. Chakrabarti *et al* had found total absence of cells in the CSF of 50% of patients in their study[49]. It is well known that the CSF findings in CM can be non-specific. But sometimes lowered glucose levels or elevated protein/ cell counts are encountered.

The positivity rate of detection of *Cryptococcus* in the CSF varied between the diagnostic tests used in this study (Table 7). Among the 16 patients who presented with primary episode of CM, India ink preparation was positive in 11 (68.75%), LAT in 16 (100%) and culture in 12 (75%). Gram stain however, showed budding yeast like organisms (YLO) in only 7 (43.75%) patients. In a study by Imwidthaya *et al*[37], the positivity of India ink, LAT and culture for *Cryptococcus* were 91%, 100% and 100% respectively. However, Nigam *et al*[70] had found positivity rate of 70% (India ink), 100% (LAT) and 85% (culture) which is comparable to the results obtained in this study.

Among the 4 patients whose culture was negative for *Cryptococcus*, 2 were started on empiric amphotericin-B before lumbar puncture was performed. This was done in the interest of the patients as it is well known that the disease is associated with a high mortality rate. In the other 2 patients, the exact reason for failure to grow the yeast could not be exactly ascertained. It is likely that the organism burden in the CSF of these patients could have been low or an insufficient volume of CSF was cultured.

In this study, there were 3 relapse cases accounting for 15.79% of the total cases admitted with CM. In a study by Jarvis *et al*, relapse was seen in 23% of CM cases admitted to their hospital[40]. Among the 3 patients who presented with a relapse episode, India ink, LAT & culture was positive in 2 (66.67%), 3 (100%) and 1 (33.33%) respectively. In this study, the first patient among the 3 who had an episode of relapse had discontinued secondary prophylaxis and presented with a relapse 4 months later. During

this episode, he succumbed to the infection. It is estimated that without secondary prophylaxis, atleast 50-60% of patients with CM will have disease relapse[19.98]. When administered properly, secondary prophylaxis with fluconazole is highly effective, increasing recurrence free survival by over thirteen fold at six months[13, 80]. In this study, the other 2 patients with relapse had received fluconazole prophylaxis. HAART was started 6 weeks after the primary episode in one patient and after 8 weeks in the other patient. So, the relapse in these 2 patients was due to IRIS. Therefore, IRIS accounted for 10.5% of the total cases admitted with CM in this study. The former patient presented with IRIS after 43 days of starting HAART while the latter developed IRIS after 135 days of starting HAART. The median duration between starting HAART and development of IRIS in these patients was 89 days. India ink was positive in one patient while culture for *Cryptococcus* was negative in both these patients. There was no mortality observed and both of them are still under follow up. In a study by Jarvis *et al*, IRIS accounted for 10.3% of all CM cases occurring at a median of 61 days after commencement of HAART[40]. In Aslam *et al* study, 4% of patients presented with IRIS which occurred at a median of 120 days after initiation of HAART [5]. A multicentre prospective study published in 2009 concluded that a large proportion of HIV positive patients with CM may develop an IRIS illness after starting HIV treatment but the increased likelihood of successful outcome when using HAART could outweigh the risks of developing IRIS in these patients.

In this study, culture of CSF on SDA showed mucoid, cream coloured, yeast like colonies within 48 hours in 2 samples while 9 samples grew yeast like colonies in 48-72 hours (Table 8). In the remaining 2 culture positive samples, growth was observed on the 5th and 7th day respectively. Delayed growth of *Cryptococcus* varying from 5-14 days has been previously noted in other studies as well[42, 45].

In this study, urea was hydrolysed within 24 hours on Christensen's urease medium by all the 13 cryptococci isolated from CSF of patients with CM (Table 9). In addition, all these isolates also formed brown colonies on CFA medium. The duration for production of pigment varied from 2-4 days. For speciating the cryptococci, the isolates were subcultured on CGB medium and also tested for their ability to assimilate proline. 12 out of the 13 cryptococci isolated in this study neither grew on CGB medium nor assimilated proline indicating that they were *C.neoformans*. There are only few reports of *C.gattii* causing meningitis in HIV patients, and in this study, *C.gattii* was isolated from a single patient (7.69%). In most of the studies including that by Baradkar *et al* [6] and Manoharan *et al* [63], all the cryptococcal isolates were *C.neoformans*. However, Nigam *et al* found that5.88% of cryptococci isolated from HIV positive patients in their study were *C.gattii* [70]. For determining the varietal status, the 12 isolates of *C.neoformans* were also subcultured on CDBT medium. All the 12 isolates failed to grow on this medium indicating their inability to assimilate thymine. Therefore, all the isolates in this study were found to be *C.neoformans* var. *grubii*. Both, in India and globally, greater than 90% of cryptococcal infections in HIV patients are caused by *C.neoformans* var. *grubii* (serotype A) [65]. The findings of this study were therefore, consistent with those of the earlier ones.

The result of assimilation of carbohydrates showed that all 12 *C.neoformans* isolates assimilated inositol, dextrose, maltose and sucrose but none of them assimilated lactose. Cellobiose, however, was assimilated by only 10 of the isolates (Table 10). The *C.gattii* isolate assimilated inositol, dextrose, maltose and sucrose but not lactose and cellobiose. It is well known that *C.neoformans* and *C.gattii* do not assimilate lactose. Assimilation of cellobiose, however, varies between individual strains[27].

Due to scarce availability of 5-flucytosine (5-FC), none of the patients in this study received amphotericin-B + 5-FC in combination for induction phase therapy of CM (Table 11). But 15 of the 16 patients who presented with primary episode of CM received intravenous amphotericin-B, 6 in the form of monotherapy and 9 in combination with oral fluconazole. One patient expired before a diagnosis of CM was made and had only received intravenous ceftriaxone. However, despite institution of amphotericin-B, 6 patients died during the primary episode itself. The mortality rate observed for primary episodes of CM was 43.75%. The remaining 9 patients were started on secondary fluconazole prophylaxis but one patient discontinued it and presented with relapse later and died. So, overall mortality of 42.1% due to CM was observed in this study. This is higher when compared to mortality rate of 25.64% found by Lakshmi *et al* [55] and 15% found by Nigam *et al* [70] in their studies respectively.

Estimation of the CD4 count at the time of presentation with primary episode of CM showed that 14 out of 15 patients had counts <100cells/µl and only one patient had a count of 115cells/µl (chart 8). One patient died before estimation of the CD4 count. In this study, the mean CD4 count of patients who survived the infection was 71.63cells/µl while the mean CD4 count of patients who succumbed to the infection was 34.71cells/µl. However, both Aslam *et al* [5] and Mahale *et al* [61] had found mean CD4 count of 60cells/µl in their studies. It was observed that 75% of patients with CD4 count <40cells/µl died while only 14.28% of patients with CD4 count $\geq$40cells/µl died (Table 12). The association between death of patients with CD4 count <40cells/µl compared to those with CD4 counts $\geq$40cells/µl was found to be statistically significant ($p = 0.018$). Among the 3 patients who had an episode of relapse, one showed deterioration in CD4 count from 63 to 32 while the other 2 patients documented a rise in CD4 count from 56 to 151 and 9 to 89

respectively. The two patients with rise in CD4 counts had relapse due to IRIS and survived the episode but the other patient died.

Antifungal susceptibility testing was performed by microbroth dilution reference method of CLSI as per M27-A3 protocol[20]. MIC of amphotericin-B, fluconazole and voriconazole was determined for all the isolates obtained in CSF culture. All the *C.neoformans* and *C.gattii* were sensitive to amphotericin-B with MIC ranging from 0.12-0.5µg/ml using microbroth dilution method (Table 13) and 0.016-0.256µg/ml using epsilometer test (Table 14). Isolates of *C.neoformans* had relatively lower MIC values with the E test for amphotericin-B when compared with the microbroth dilution method (Table 15). Significant association (p=0.002) was observed between testing cryptococcal isolates by E test and obtaining lower MIC values ≤0.12µg/ml for amphotericin-B. Essential agreement (EA) between MIC values obtained by E test when compared with the reference microbroth dilution test was 61.5% for amphotericin-B in this study. In a study by Tewari *et al* [91], EA of 76% for MIC of amphotericin-B obtained by E test when compared to microbroth dilution test was found against 62 Indian clinical isolates of *C.neoformans* var.*grubii*. However, categorical agreement of 100% was observed both in this study and in the one conducted by Tewari *et al* [91].

All the *C.neoformans* and *C.gattii* were also sensitive to fluconazole with MIC ranging from 0.5- 2µg/ml using microbroth dilution method (Table 16) and 1-8µg/ml using epsilometer test (Table 17). However, in contrast to amphotericin-B, isolates of both *C.neoformans* and *C.gattii* exhibited relatively higher MIC values with the E test for fluconazole when compared with the microbroth dilution method (Table 18). Significant association (p=0.008) was observed between testing cryptococcal isolates by E test and obtaining higher MIC values ≥4µg/ml for fluconazole. Essential agreement of 76.9% for MIC values of fluconazole between E test and reference method was found in this study.

Tewari *et al* [91]had found EA of 82.2% for fluconazole in their study. Categorical agreement (CA) of 100% for fluconazole was observed in this study while Tewari *et al* [91] had found CA of only 77.4% between E test and reference method for fluconazole in their study.The variations in MIC values obtained with the E test for both amphotericin-B and fluconazole make it imperative that cryptococcal isolates be tested with the microbroth dilution method as recommended by the CLSI.

All cryptococcal isolates were sensitive to voriconazole too and the MIC values ranged from 0.06-0.25µg/ml (Table 19).The sensitivity of cryptococcal isolates to antifungal agents was found to be 100% in this study (Table 20). As of now, resistance of *C.neoformans* to antifungal agents is very rare[4, 11, 67] and even in the global scenario, only sporadic reports of resistance to fluconazole have been documented[18, 48].

Summary

SUMMARY

A total of 175 HIV positive patients presenting with features of meningitis were included in this study. 19 of them were found to have cryptococcal meningitis of which 16 were primary episodes and 3 were episodes of relapse. The prevalence of cryptococcal meningitis was 10.58%. Majority of patients were males (81.25%) with a male to female ratio of 5.3:1. Most of the affected patients were in the age group of 41-50 years.

Headache was the predominant presenting symptom occurring in 89.47% of patients followed by vomiting in 63.16% and fever in 42.11% of patients. Altered sensorium and neck stiffness were seen in 36.84% of patients each. Blurring of vision and motor deficits were observed in 21.05% and 15.79% of patients respectively. Papilledema was noted in 10.53% of patients. One patient each had an episode of seizure and urinary incontinence respectively. In addition to features of meningitis, two patients presented with disseminated infection of whom one patient had papulovesicular skin lesions and the other had osteomyelitis of the left 1st metacarpal bone.

The time between diagnosis of HIV infection and presentation with primary episode of cryptococcal meningitis was between 1- 2 years in most of the patients. CM was the AIDS defining illness in 3 patients. Moreover, 9 patients were on ART at the time of presentation with first episode of CM and 3 patients were also taking T. fluconazole in addition to ART.

Biochemical analysis of CSF in cases of CM showed normal glucose levels in 63.15% of patients. Elevated protein levels were seen in 73.68% of patients and elevated cell counts were observed in 78.94% of patients. Lymphocytes were the predominant cell type observed. Total absence of cells in the CSF was noticed in 2 patients.

Among patients who presented with primary episode of CM, India ink preparation was positive in 11 (68.75%), LAT was positive in 16 (100%) and culture was positive in 12 (75 %). Among patients presenting with relapse, India ink preparation was positive in 2 (66.67%), LAT was positive in 3 (100%) and culture was positive in 1 (33.33%). Growth of *Cryptococcus* on SDA was observed within 72 hours in all except two CSF samples which grew on the 5^{th} and 7^{th} day respectively.

Relapse episodes in 2 patients were due to IRIS occurring at a median of 89 days after the commencement of HAART. One patient developed relapse due to discontinuation of secondary fluconazole prophylaxis.

All the 13 isolates of *Cryptococcus* obtained from culture of CSF hydrolysed urea and produced brown colonies on CFA. Of the 13 cryptococcal isolates, 12 were *C.neoformans* and only one was *C.gattii*. The variety differentiation on CDBT medium showed that all the 12 *C.neoformans* isolated in this study belonged to *C.neoformans* var. *grubii.*

All the 12 *C.neoformans* isolates assimilated inositol, dextrose, maltose and sucrose but none of them assimilated lactose. Cellobiose, however, was assimilated by only 10 of the isolates. The *C.gattii* isolate assimilated inositol, dextrose, maltose and sucrose but not lactose and cellobiose.

In the treatment of primary episode of CM, none of the patients received combination therapy of amphotericin-B with 5-flucytosine. But, 6 patients received intravenous amphotericin-B as monotherapy while 9 patients received intravenous amphotericin-B and oral fluconazole in combination. One patient died before starting amphotericin-B itself while 6 more patients died despite institution of amphotericin-B. In

addition, another patient died during the relapse episode. The overall mortality rate observed was 42.1%.

All except one patient had CD4 count <100cells/µl at the time of presentation with primary episode of CM. The mean CD4 count of patients who survived the infection was 71.63cells/µl while the mean CD4 count of patients who succumbed to the infection was 34.71cells/µl. The association between death of patients with CD4 count <40cells/µl compared to those with counts ≥40cells/µl was found to be statistically significant ($p = 0.018$). Two patients who had relapse due to IRIS showed elevated CD4 counts compared to their respective count in the primary episode.

Antifungal susceptibility testing performed by microbroth dilution method showed that all *C.neoformans* and *C.gattii* isolates were susceptible to the antifungal agents tested. For amphotericin-B, the MIC values ranged from 0.12- 0.5µg/ml using microbroth dilution method and 0.016-0.256µg/ml using epsilometer test. Significant association ($p=0.002$) was observed between testing cryptococcal isolates by E test and obtaining lower MIC values ≤0.12µg/ml for amphotericin-B. Essential agreement (EA) of 61.5% and Categorical agreement (CA) of 100% for amphotericin-B was found in this study. For fluconazole, the MIC values ranged from 0.5- 2µg/ml using microbroth dilution method and 1-8µg/ml using epsilometer test. Significant association ($p=0.008$) was observed between testing cryptococcal isolates by E test and obtaining higher MIC values ≥4µg/ml for fluconazole. EA of fluconazole was 76.9% while the CA was 100%.The MIC values ranged from 0.06-0.25µg/ml for voriconazole by microbroth dilution method.

Conclusion

CONCLUSION

There is a decline in the number of cases of cryptococcal meningitis due to better diagnostic facilities for early detection of HIV infection and wider accessibility to ART. Male patients are more often affected and the symptoms of CM overlap with those due to other pathogens. Microscopic examination of CSF by India ink preparation is cost effective in the diagnosis of CM in HIV positive patients. LAT is highly sensitive in detecting cryptococcal polysaccharide in laboratories where the facility is available. Culture of CSF is the gold standard test in the diagnosis of CM and must be performed on all suspected cases of meningitis. *C.neoformans* is more often the etiologic agent of CM in HIV positive patients when compared to *C.gattii*. Treatment of patients with CM using amphotericin-B at the earliest is needed to reduce the mortality which is still unacceptably high. Secondary fluconazole prophylaxis must be started in all patients with CM to reduce/prevent the occurrence of relapse. Institution of HAART must be done after treatment of the primary episode as the benefits outweigh the risk of development of IRIS in these patients. CM often occurs when CD4 count falls below 100cells/µl. So, initiation of ART at CD4 count of 350cells/µl as recommended by the recent NACO guidelines can go a long way in further reduction of incidence of CM. Although resistance of *C.neoformans* to antifungal agents is rare, periodic monitoring of MIC by microbroth dilution tests is essential to notice any shifts in sensitivity pattern of clinical isolates.

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ABBREVIATIONS

AIDS Acquired Immuno Deficiency Syndrome

ART- Anti Retroviral Therapy

ATCC- American Type Culture Collection

BAL- BronchoAlveolar Lavage

CD4- Cluster of Differentiation 4

CDBT- Creatinine Dextrose Bromothymol blue Thymine

CFA- Caffeic acid Ferric citrate agar

CGB- Canavanine Glycine Bromothymol blue

CLSI- Clinical and Laboratory Standards Institute

CM- Cryptococcal Meningitis

CNS- Central Nervous System

CRAG- Cryptococcal Antigen

CSF- Cerebrospinal fluid

CT- Computed Tomography

EIA- Enzyme Immunoassay

GTCS- Generalised Tonic Clonic Seizures

HAART- Highly Active Anti Retroviral Therapy

HIV- Human Immunodeficiency Virus

ICT- Intracranial Tension

IRIS- Immune Reconstitution Inflammatory Syndrome

KOH- Potassium hydroxide

LAT- Latex Agglutination Test

LCB- Lactophenol Cotton Blue

MIC- Minimum Inhibitory Concentration

MRI- Magnetic Resonance Imaging

NACO- National AIDS Control Organisation

NACP- National AIDS Control Program

QC- Quality Control

RPMI- Rosewell Park Memorial Institute

SDA- Sabouraud Dextrose Agar

YCB- Yeast Carbon Base

YLO- Yeast like Organism

YNB- Yeast Nitrogen Base

APPENDIX- I

(STAINS, REAGENTS AND MEDIA)

I. GRAM STAINING

Methyl violet (2%)	l0g Methyl violet in l00ml absolute alcohol in 1litre of distilled water (primary stain)
Gram's Iodine	l0g Iodine in 20g KI (fixative)
Acetone	(decolourising agent)
Carbol fuchsin 1%	(counter stain)

II. INDIA INK

India ink	150ml
Merthiolate (1:1000)	3ml
Tween 80 (1: 10,000)	0.1ml

III. 10% KOH

Potassium hydroxide	10 g
Glycerol	10 ml
Distilled water	80 ml

1. SABOURAUD DEXTROSE AGAR (SDA)

Glucose	40gm
Peptone	10gm
Agar	20gm
Distilled water	1000ml

Mix the reagents by boiling. Dispense in tubes and autoclave at 121^0C for 15 minutes. Final pH must be 5.5 – 5.6. Allow the tubes to cool in slanted position.

2. CHRISTENSEN'S UREASE TEST MEDIUM

Peptone	1g
Sodium chloride	5g
Dipotassium hydrogen phosphate	2g
Phenol red	6ml
Agar	20g
Distilled water	1 ltr
10% sterile solution of glucose	l0ml
Sterile 20% urea solution	l00ml

Sterilize the glucose and urea solutions by filtration. Prepare the basal medium without glucose and urea, adjust to pH 6.8-6.9 and sterilize by autoclaving in a flask at 121°C for 30min. Cool to about 50°C, add the glucose & urea, and tube the medium as slopes.

3. CAFFEIC ACID FERRIC CITRATE MEDIUM

Ingredient	gm/ltr
Yeast extract	2
Dextrose	5
Ammonium sulphate	5
Dipotassium phosphate	5
Magnesium sulphate	0.7
Caffeic acid	0.18
Ferric citrate	0.02
Agar	20

pH 6.5 $\pm$ 0.2

Suspend 33.7 grams in 1 litre of distilled water. Heat until the solution boils to dissolve the contents completely. Sterilize by autoclaving at 121^0C for 15 minutes. Cool to 55^0C, add chloramphenicol 50µg/ml and dispense in plates/ tubes

4. CANAVANINE GLYCINE BROMOTHYMOL BLUE (CGB) MEDIUM

Solution A

Glycine	10gm
Potassium dihydrogen orthophosphate	1gm
Magnesium sulphate	1gm
L- canavanine sulphate	30µg
Thiamine hydrochloride	1µg
Distilled water	100ml

Adjust pH to 5.6 $\pm$ 0.2 and filter sterilize

Solution B

Bromothymol blue	0.4gm
Sodium hydroxide (NaOH) 0.01M	64ml
Distilled water	36ml

Add 2ml of solution B to 88ml of distilled water. Add 2 gms agar and autoclave at 121^0C for 15 minutes. Cool to 55^0C and add 10ml of solution A. Mix well and pour in tubes/plates.

5. CREATININE DEXTROSE BROMOTHYMOL BLUE THYMINE (CDBT) MEDIUM

Solution A

Creatinine	1gm
Potassium dihydrogen orthophosphate	1gm
Magnesium sulphate	0.5gm
Dextrose	0.5gm
Thymine	0.1gm
Distilled water	980ml

Adjust pH to 5.6 and filter sterilize

Solution B

Bromothymol blue	0.4gm
Sodium hydroxide (NaOH) 0.01M	64ml
Distilled water	36ml

pH: 5.6 $\pm$ 0.2

Mix solution A and 20ml of solution B. Add 20 gms agar and autoclave at 121^0C for 15 minutes. Cool to 55^0C and pour in plates.

APPENDIX- II

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI -3

Telephone No : 04425305301
Fax : 04425363970

CERTIFICATE OF APPROVAL

To
Dr. J. Durdana Parveen
PG in MD Microbiology
Madras Medical College, Chennai -3

Dear Dr. J. Durdana Parveen

The Institutional Ethics Committee of Madras Medical College reviewed and discussed your application for approval of the proposal entitled " Cryptococcal meningitis in immunocompromised patients with special reference to AIDS " No.230902011

The following members of Ethics Committee were present in the meeting held on 27.09.2011 conducted at Madras Medical College, Chennai –3

1. Dr. S.K. Rajan MD	-- Chairperson
2. Dr. V. Kanagasabai MD Dean, Madras Medical College, Chennai -3	-- Deputy Chairman
3. Prof. R. Sundaram MD Vice Principal, Madras Medical College, Chennai -3	-- Member Secretary
4. Prof. R. Nandhini MD Director , Inst. of Pharmacology, MMC , Ch-3	-- Member
5. Prof. Pregna B. Dolia MD Director , Inst. of Biochemistry, M M C, Ch-3	-- Member
6. Thiru . Ulaganathan Administrative Officer, M M C, Ch-3	-- Layperson
7. Thiru. S. Govindasamy BA BL	-- Lawyer
8. Tmt. Arnold Saulina .MA., MSW	-- Social Scientist

We approve the Proposal to be conducted in its presented from

Sd/ Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study any SAE occurring in the course of the study , any changes in the protocol and patient information / informed consent and asks to be provided a copy of the final report.

Member Secretary, Ethics committee

Govt. Hospital of Thoracic Medicine

Tambaram Sanatorium Chennai-47, Tamilnadu, India

Phone : (91-044-22418450/22418427)

Fax : (91-044-22418668)

Dr. O. R. Krishnarajasekhar, MD.DTRD.,
Deputy Superintendent and Member Secretary,
Institutional Review Board,
GHTM.

To
Dr. Dr. Durdana Parveen.J
M.D. Microbiology- II yr Postgraduate,
Madras Medical College,
Institute of Microbiology,
Madras Medical College,
Chennai-600 003

Madam,

The research project entitled "A study on cryptococcal meningitis in patients with HIV/AIDS" has been approved by the Institutional Review Board Committee of GHTM during its meeting on 13th January 2012.

With regards,

ORKrishnarajasekhar 08/02/2012
Dr. O. R. Krishnarajasekhar,
Member-Secretary

Place : Chennai
Date : 8th February 2012

GHTM
INSTITUTIONAL REVIEW BOARD

APPENDIX- III (PROFORMA)

- Name : IP no:
- Age/ Sex: Ward:
- Occupation:
- Address:

- Presenting complaints:

COMPLAINT	DURATION
Fever	
Headache	
Nausea/vomiting	
Drowsiness	
Visual disturbances	
Others	

- Past history of similar episode:
- H/O T.Fluconazole administration?

- HIV status & duration:
- Whether on ART:
- CD_4 count (at the time of presentation with meningitis) :

- <u>Associated comorbid illness</u>

- <u>Physical examination:</u>

  Fever
  Neck stiffness
  Altered sensorium
  Signs of meningeal irritation
  Focal neurological deficit
  Papilledema (Fundus examination)

- Treatment received by the patient for the current episode:
- Follow up:

¤ Laboratory Evaluation:

CSF Sugar
CSF Protein
CSF Cell count

India ink preparation
Gram stain

Latex Agglutination Test (LAT)

Culture- SDA

- Caffeic acid agar
- CGB medium
- CDBT medium

Urea hydrolysis

Assimilation of carbohydrates

- Dextrose
- Inositol
- Lactose
- Maltose
- Sucrose
- Cellobiose

Antifungal susceptibility testing

	BROTH DILUTION		**E TEST**	
	MIC	**S/S-DD/R**	**MIC**	**S/S-DD/R**
Amphotericin-B				
Fluconazole				
Voriconazole			-	-

APPENDIX IV- PATIENT CONSENT FORM

சுய ஒப்புதல் படிவம்

ஆய்வு செய்யப்படும் தலைப்பு:

நோய் எதிர்ப்பு தன்மை குறைந்து மூளைக் காய்ச்சலால் பாதிக்கப்பட்டவர்களுக்கு குறிப்பாக எய்ட்ஸ் நோயாளிகளுக்கு கிரிப்டோகாக்கஸ் பூஞ்சை தாக்கியிருப்பதை கண்டறியும் ஆய்வு.

சென்னை மருத்துவக் கல்லூரி, நுண்ணுயிரியல் துறையில் பயிலும் முதுகலை மருத்துவர். J. துர்தானா பர்வீன், அவர்கள் மேற்கொள்ளும் இந்த ஆய்வில் பங்குகொள்ள ..... ஆகிய நான் முழுமனதுடன் சம்மதிக்கிறேன். இந்த ஆய்வை மேற்கொள்ளும் மருத்துவர் என் மருத்துவ விவரங்கள் மற்றும் மருத்துவ ஆய்வின் முடிவுகள் ஆகியவற்றை தெரிந்துகொள்ளவும், என்னுடைய தண்டு வட நீரை பரிசோதனைக்கு எடுத்துக்கொள்ளவும் முழுமனதுடன் சம்மதிக்கிறேன். இந்த ஆய்வினால் எந்த தீங்கும் ஏற்படாது என்பதையும் அறிவேன். மேலும் இந்த ஆய்வின் முடிவுகளை பிரசுரிக்கவும் சம்மதிக்கிறேன்.

பங்கேற்பவரின் கையொப்பம்..... இடம் :..... தேதி.....
கட்டைவிரல் ரேகை

பங்கேற்பவரின் பெயர் மற்றும் விலாசம்.....

ஆய்வாளரின் கையொப்பம்.....இடம் :..... தேதி.....

ஆய்வாளரின் பெயர்.....

CRYPTOCOCCAL MENINGITIS IN HIV/AIDS PATIENTS

	O.P. No.	Age	Sex	Fever	Headache	Vomiting	Alt.sensorium	Neck stiff	Papilledema	Other symp	Associated illness	HIV dur	On ART	CD4 count	Rx T.Fluc	Relapse	Drug Rx	Dur of Rx	Follow up	CSF- S/P/C	India ink	Gram stain	LAT	SDA	Urease	CFA	CGB	CDBT	AM-B (MBD)	AM-B (E test)	FLU (MBD)	FLU (E TEST)	VOR (MBD)
1	123604/11	23	M	No	Yes	No	Yes	No	No	Cough+	PT	3years	Yes	439	No	No	Ceftriax	3days	Survived	23/234/58	Neg	moderate pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	412902/12	36	M	No	Yes	No	No	No	No	Nil	Nil	2years	No	not tested	No	No	Ceftriax	7days	Survived	73/39/0	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	155903/12	30	M	No	Yes	No	Yes	No	No	Nil	Nil	5years	yes	34	Yes	No	Amp-B	2days	Survived	10/148/65	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	285004/03	30	F	Yes	Yes	Yes	No	No	No	Nil	diarrhoea	3years	Yes	118	Yes	No	ceftriax	5days	Survived	55/37/7	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	37890	22	F	No	No	Yes	Yes	Yes	No	Cough+	PTB	2years	No	141	No	No	Ceftriax, DOTS	3days	Survived	36/20/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	398202/12	45	M	Yes	Yes	No	No	No	No	Nil	Nil	5years	yes	140	Yes	No	ceftriax	3days	Survived	41/39/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	212004/12	37	F	No	Yes	Yes	No	No	No	limb paresis	Nil	3years	Yes	85	Yes	No	ceftriax	3days	Survived	50/-/5	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
8	634306/09	30	F	Yes	Yes	Yes	No	No	No	Nil	Nil	2years	No	264	No	No	ceftriax	3days	Survived	36/20/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	184104/12	30	M	Yes	Yes	Yes	Yes	No	No	Cough, hemoptysis+	ATT default, oral thrush	3years	Yes	19	Yes	No	ceftriax	3days	Survived	55/37/7	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
10	349904/12	42	M	Yes	Yes	Yes	Yes	Yes	No	Nil	Left hemianopia	Newly diag	No	128	No	No	ceftriax	3days	Survived	10/148/65	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
11	100202/12	27	M	No	No	Yes	Yes	Yes	No	cough	PTB	2years	No	151	No	No	ceftriax	5days	Survived	30/142/48	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
12	26895	45	M	Yes	Yes	Yes	No	No	No	cough	PTB	2years	Yes	54	No	No	Amp-B	3days	Died	12/44/-	pos	pus cells+, YLO	Positive	growth (72 hrs)	Pos	Brown col	Growth +	NA	0.5	0.5	2	8	0.5
13	60506/12	32	F	No	Yes	No	Yes	No	No	Nil	Nil	6months	No	121	No	No	Ceftriax	3days	Survived	30/142/48	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
14	176306/12	38	M	Yes	No	No	Yes	No	No	Nil	Nil	2years	Yes	224	No	No	Ceftriax	5days	Survived	36/20/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	421611/2010	25	M	No	Yes	Yes	No	No	No	Nil	Nil	2years	No	267	No	No	Ceftriax	5days	Survived	32/21/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
16	157807/12	25	F	No	Yes	Yes	No	No	No	Nil	Nil	2years	Yes	198	No	No	Ceftriax	5days	Survived	18/40/I	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
17	673803/04	35	M	No	Yes	No	Yes	No	No	Nil	Nil	2years	Yes	302	No	No	Ceftriax	5days	Survived	30/142/48	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
18	542807/05	35	M	No	Yes	No	Yes	No	No	Nil	Nil	2years	Yes	121	No	No	Ceftriax	5days	Survived	36/20/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
19	148307/12	31	M	Yes	No	Yes	No	No	No	Nil	Nil	6months	No	148	No	No	Ceftriax	3days	Survived	10/148/65	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
20	48008/12	28	F	Yes	No	No	Yes	No	No	cough	Nil	2years	Yes	498	No	No	Ceftriax, DOTS	3days	Survived	23/234/58	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
21	29701	39	M	No	Yes	No	Yes	No	No	Nil	No	3 yrs	No	63	No	No	Am-B	10days	Survived	42/20/12	Pos	many pus cells, YLO	Positive	Growth (48hrs)	Pos	Brown col	NG	NG	0.25	0.12	0.5	1	0.12
22	164605/12	28	F	Yes	Yes	No	No	No	No	cough	PTB	2years	Yes	29	No	No	Ceftriax, DOTS	5days	Died	32/21/4	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
23	48008/12	28	M	No	No	Yes	Yes	No	No	Nil	Nil	2years	Yes	316	No	No	Ceftriax	3days	Survived	40/26/8	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
24	195308/12	25	M	No	Yes	No	Yes	No	No	Nil	Nil	2years	Yes	287	No	No	Ceftriax	3days	Survived	32/21/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
25	48008/12	28	F	No	No	Yes	Yes	No	No	Nil	Nil	2years	Yes	269	No	No	Ceftriax	3days	Survived	30/142/48	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
26	235804/12	28	M	Yes	Yes	Yes	Yes	Yes	No	cough dyspnea	oral thrush	4years	Yes	43	Yes	No	Ceftriax	5days	Died	23/377/-	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
27	89224	17	F	Yes	Yes	Yes	Yes	No	No	Nil	nil	3yrs	NA	not tested	NA	No	Ceftriax	5days	Survived	32/21/4	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
28	109400	14	M	No	Yes	Yes	No	Yes	No	Nil	No	14yrs	No	399	NA	No	Ceftriax	4days	Survived	46/20/8	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
29	109700	32	M	No	Yes	No	Yes	No	No	Nil	PTB	5yrs	No	410	NA	No	Ceftriax	5days	Survived	39/21/6	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
30	1009	42	M	No	Yes	No	Yes	No	No	FND+	No	3yrs	No	388	NA	No	Ceftriax	3days	Survived	46/28/2	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
31	20708	24	F	Yes	Yes	Yes	No	No	No	Nil	Nil	6months	No	397	No	No	Ceftriax	5days	Survived	48/16/5	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
32	36590	36	F	Yes	Yes	No	Yes	Yes	No	Nil	diarrhoea	2yrs	No	278	No	No	Ceftriax	5days	Survived	28/32/12	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
33	470409/05	31	M	No	Yes	Yes	No	No	No	Cough+	PT	7years	Yes	208	No	No	Cat II DOTS, Amp-B	5days	Survived	30/183/18	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
34	367512/10	30	M	No	Yes	No	No	No	No	Nil	Nil	2years	No	659	No	No	Ceftriax	5days	Survived	46/16/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
35	393302/12	29	F	Yes	Yes	No	No	No	No	Nil	Nil	2years	No	not tested	No	No	Ceftriax	5days	Survived	30/139/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
36	67703/05	29	M	Yes	Yes	No	No	No	No	Cough+	PT	7years	Yes	11	Yes	No	Amp-B	3days	Died	60/64/38	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA

37	203303/07	36	M	Yes	Yes	Yes	No	No	No	Cough+	Oral ulcers, Thrush, Cerv. LAD	5years	Yes	618	Yes	No	Cat I DOTS	5days	Survived	64/28/6	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
38	91488	65	M	Yes	Yes	Yes	Yes	Yes	No	Thrush	DM	2yrs	Yes	176	NA	No	Ceftriax	1day	Died	16/34/l	Neg	many pus cells,gpc pairs	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA
39	185412/11	28	M	Yes	Yes	No	No	No	No	Nil	Nil	3yrs	yes	56	no	No	Amp-B	10days	Survived	36/82/22	pos	pus cells+, YLO	Positive	growth (24 hrs)	Pos	Brown col	NG	NA	0.25	0.12	1	2	0.12
40	87903/11	41	M	No	No	Yes	Yes	No	No	cough	pleural effusion	3yrs	No	411	No	No	Ceftriax, DOTS	5days	Survived	30/142/48	Neg	occ. pus cells	Neg		NA	NA	NA	NA	NA	NA	NA	NA	NA
41	47103/12	41	M	Yes	No	No	Yes	No	No	Cough+	Thrush+	2years	Yes	299	Yes	No	Amp-B	2days	Survived	48/65/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
42	420602/12	55	M	Yes	No	No	Yes	No	No	Cough+	B/L nephropathy	Newly diag	No	74	Yes	No	Amp-B	1day	Survived	85/24/0	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
43	144904/11	35	M	No	Yes	No	Yes	No	No	Nil	Nil	2years	Yes	99	No	No	Cat I DOTS	5days	Survived	13/227/108	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
44	29701	39	M	Yes	Yes	Yes	No	Yes	No	skin lesions	oral thrush	3 yrs	No	32	default	Yes	Amp-B	2days	Died	38/40/11	pos	many pus cells, YLO	Positive	Growth (48 hrs)	Pos	Brown col	NG	NG	0.25	0.12	0.5	4	0.06
45	95003/12	45	M	Yes	Yes	No	No	No	No	Nil	Nil	3years	Yes	28	Yes	No	Ceftriax	5days	Survived	53/48/42	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
46	147406/12	45	M	No	No	Yes	Yes	No	No	cough	Nil	3years	No	172	No	No	ceftriax	5days	Survived	32/21/4	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
47	214903/08	37	M	Yes	No	Yes	No	No	No	cough	Nil	3years	No	213	No	No	ceftriax	5days	Survived	40/26/8	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
48	224203/12	33	M	Yes	Yes	No	Yes	No	No	Cough, hemoptysis+	PT with PE, Abd TB, Oral ulcers,thrush	1year	No	not tested	Yes	No	Ceftriax, DOTS	3days	Died	32/31/2.3	Neg	moderate pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
49	100403/12	42	M	No	Yes	No	No	No	No	Nil	Nil	1year	No	338	No	No	ceftriax	3days	Survived	24/42/4	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
50	116702/11	41	M	Yes	Yes	No	No	No	No	Nil	diarrhoea	2years	yes	137	No	No	cotrimox	3days	Survived	59/33/12	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
51	290403/12	45	M	No	Yes	Yes	No	No	No	Nil	Nil	2years	No	262	No	No	ceftriax	7days	Survived	30/142/48	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
52	306503/12	27	M	Yes	Yes	Yes	No	No	No	Nil	ATT default	2years	yes	115	Yes	No	ceftriax	7days	Survived	40/102/-	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
53	304203/12	33	M	Yes	Yes	Yes	No	Yes	No	limb paresis, GTCS	Oral ulcers, Thrush	5years	yes	59	Yes	No	Amp-B	6days	Died	49/38/22	Neg	occ. pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
54	159103/12	37	M	No	Yes	Yes	No	No	No	Nil	Nil	2years	No	not tested	No	No	ceftriax	7days	Died	52/48/12	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
55	136302/12	31	F	Yes	Yes	Yes	No	No	No	Nil	Nil	4years	yes	42	No	No	Amp-B	10 days	Survived	59/53/11	Neg	moderate pus cells	Positive	NG	NA	NA	NA	NA	NA	NA	NA	NA
56	160004/12	22	M	Yes	Yes	Yes	No	No	No	Nil	Nil	1year	No	not tested	No	No	ceftriax	2days	Died	30/142/48	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
57	160604/12	35	M	No	Yes	Yes	No	No	No	Nil	Nil	4years	Yes	110	Yes	No	ceftriax	7days	Survived	40/26/8	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
58	195606/12	33	M	Yes	No	No	Yes	No	No	cough	PTB	6months	No	235	No	No	ceftriax	5days	Survived	59/33/12	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
59	78306/08	32	M	No	Yes	Yes	No	No	No	FND+	On ATT	1year	No	333	No	No	ceftriax	7days	Survived	71/38/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
60	344906/11	30	M	Yes	Yes	Yes	No	No	No	Nil	Nil	2years	No	298	No	No	ceftriax	7days	Survived	41/86/0	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
61	76106/12	38	M	Yes	No	No	Yes	Yes	No	cough	PTB	3years	Yes	31	No	No	ceftriax	5days	Died	59/33/12	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
62	32485	38	F	No	Yes	Yes	Yes	No	No	Blurring of vision	PTB	1yr	Yes	35	No	No	Am-B	2days	Died	40/26/8	Pos	moderate pus cells	Positive	Growth (48hrs)	Pos	Brown col	NG	NG	0.25	0.25	1	2	0.12
63	1003/11	47	M	Yes	Yes	Yes	No	No	No	cough dyspnea	On ATT	3years	Yes	129	Yes	No	Ceftriax, DOTS	5days	Survived	23/234/58	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
64	306503/12	27	F	No	Yes	Yes	Yes	No	No	Nil	On ATT	2years	No	115	No	No	Ceftriax, DOTS	7days	Survived	70/101/50	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
65	136206/11	43	M	Yes	Yes	Yes	No	No	No	cough dyspnea	oral thrush	4years	Yes	not tested	No	No	Ceftriax	5days	Died	not tested	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
66	116181	33	M	No	Yes	No	Yes	Yes	No	paraparesis	oral thrush	On adm	No	34	No	No	Ceftriax	2days	Died	40/20/4	Neg	Many pus cells	Positive	Growth (72 hrs)	Pos	brown col	NG	NG	0.5	0.12	0.5	1	0.12
67	393001/07	34	M	Yes	Yes	Yes	No	No	No	FND+	ATT default, oral thrush	3years	Yes	51	Yes	No	Ceftriax, DOTS	7days	Died	23/234/58	Neg	pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
68	73807/10	38	M	Yes	No	No	Yes	No	No	cough dyspnea	ATT default, oral thrush	4years	Yes	40	Yes	No	Ceftriax, DOTS	5days	Died	65/329/8	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
69	263304/12	34	M	Yes	Yes	Yes	No	No	No	Cough, hemoptysis+	PT with PE, Oral ulcers, Thrush	4years	Yes	not tested	Yes	No	Ceftriax	5days	Died	43/65/3	Neg	pus cells+	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
70	162604/12	28	M	No	Yes	Yes	Yes	No	No	Nil	Nil	Newly diag	No	89	No	No	Ceftriax	7days	Survived	23/234/58	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
71	266404/12	30	M	No	Yes	Yes	No	No	No	FND+	On ATT	2years	No	41	Yes	No	Ceftriax, DOTS	7days	Died	59/33/12	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA
72	268204/12	55	M	Yes	Yes	No	No	No	No	Nil	Nil	3years	Yes	141	No	No	Ceftriax	3days	Survived	40/20/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA

73	181604/12	38	M	Yes	Yes	Yes	No	No	No	Nil	Nil	3years	Yes	189	No	No	Ceftriax	5days	Survived	23/234/58	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
74	316404/12	28	F	No	Yes	No	Yes	No	No	Nil	On ATT	3years	Yes	208	No	No	Ceftriax, DOTS	7days	Survived	43/65/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
75	331604/12	26	F	Yes	Yes	Yes	No	No	No	Nil	On ATT	3years	Yes	306	No	No	Ceftriax, DOTS	7days	Survived	59/33/12	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
76	303504/12	30	M	No	Yes	Yes	No	No	No	FND+	ATT default	6months	No	33	No	No	Ceftriax, DOTS	7days	Died	40/20/4	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
77	55107/02	29	M	Yes	Yes	No	No	No	No	Nil	diarrhoea	10years	Yes	125	Yes	No	Ceftriax	5days	Survived	61/50/24	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
78	19205/12	30	M	No	Yes	Yes	No	No	No	FND+	On ATT	2years	No	221	No	No	Ceftriax, DOTS	5days	Survived	23/234/58	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
79	185412/11	28	M	Yes	Yes	Yes	Yes	No	No	Nil	Nil	3years	Yes	151	Yes	Yes	Amp-B	7days	Survived	30/142/48	Positive	Occ pus cells	Positive	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
80	1003/11	47	M	Yes	Yes	Yes	No	No	No	cough dyspnea	On ATT	3years	Yes	129	Yes	No	Ceftriax, DOTS	5days	Survived	36/20/-	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
81	226205/12	35	M	No	No	Yes	Yes	No	No	cough	PTB	2years	Yes	50	No	No	Ceftriax, DOTS	5days	Died	59/33/12	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
82	303504/12	30	M	Yes	No	No	Yes	No	No	Nil	Nil	2years	Yes	455	No	No	Ceftriax	5days	Survived	23/234/58	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
83	182405/12	26	M	Yes	No	Yes	No	No	No	Nil	Nil	6months	No	128	No	No	Ceftriax	5days	Died	26/24/8	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
84	273705/12	39	F	No	No	Yes	Yes	No	No	Nil	Nil	4years	Yes	290	Yes	No	Ceftriax	5days	Survived	43/65/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
85	300805/12	43	M	Yes	No	No	No	No	No	Nil	Nil	3yrs	No	67	No	No	Ceftriax	5days	Died	10/148/65	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
86	36206/12	38	M	No	No	Yes	Yes	No	No	Nil	Nil	2years	Yes	31	No	No	Ceftriax	5days	Died	40/20/4	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
87	1003/11	47	M	Yes	No	No	Yes	Yes	No	Nil	Nil	3yrs	No	106	No	No	Ceftriax	7days	Survived	23/234/58	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
88	362908/08	43	M	No	Yes	Yes	No	No	No	Nil	Nil	5years	No	9	Yes	No	Amp-B	17days	Survived	36/70/24	Positive	Occ pus cells, YLO	Positive	growth (24 hrs)	Pos	brown col	NG	NG	0.25	0.032	1	2	0.12
89	296002/10	39	M	No	No	Yes	Yes	No	No	cough	PTB	on adm	No	128	No	No	Ceftriax, DOTS	10days	Survived	36/20/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
90	361005/12	39	M	No	Yes	No	Yes	No	No	Nil	Nil	3yrs	Yes	277	No	No	Ceftriax	7days	Survived	26/24/8	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
91	78106/12	30	M	Yes	No	Yes	No	No	No	Nil	Nil	3yrs	Yes	not tested	No	No	Ceftriax	3days	Died	23/234/58	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
92	160206/12	31	F	No	No	Yes	Yes	No	No	Nil	Nil	3yrs	Yes	243	No	No	Ceftriax	5days	Survived	40/20/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
93	508108/05	37	M	No	Yes	Yes	No	Yes	No	Nil	ATT default	7years	Yes	32	Yes	No	Amp-B	10days	Died	56/130/28	Positive	occ. pus cells+	Positive	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
94	168706/12	28	M	No	No	Yes	Yes	No	No	cough	PTB	4years	Yes	216	Yes	No	Ceftriax, DOTS	10days	Survived	18/40/l	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
95	176506/12	27	M	Yes	No	No	Yes	No	No	Nil	Nil	2years	Yes	300	No	No	Ceftriax	3days	Survived	43/65/3	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
96	155706/12	38	M	No	No	Yes	Yes	Yes	No	Nil	Nil	3yrs	Yes	296	No	No	Ceftriax	5days	Survived	36/20/-	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
97	166206/12	36	M	Yes	No	No	Yes	No	No	Nil	Nil	3yrs	Yes	207	No	No	Ceftriax	3days	Survived	26/24/8	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
98	376705/12	35	M	No	Yes	No	Yes	No	No	Nil	Nil	3yrs	Yes	149	No	No	Ceftriax	5days	Survived	40/20/4	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
99	130706/12	35	M	No	No	Yes	Yes	No	No	cough	PTB	4years	Yes	156	Yes	No	Ceftriax, DOTS	10days	Survived	23/234/58	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
100	174403/12	34	M	No	Yes	yes	No	Yes	No	visual dist+	Thrush, Eso candidiasis+	2mon	Yes, 1mon	31	No	No	Amp-B	4days	Died	29/132/28	Positive	moderate pus cells	Positive	Growth (48 hrs)	Pos	brown col	NG	NG	0.25	0.064	0.5	8	0.12
101	176506/12	26	M	No	No	Yes	Yes	No	No	cough	PTB	4years	No	98	Yes	No	Ceftriax, DOTS	10days	Survived	43/65/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
102	250506/12	24	M	Yes	No	No	Yes	No	No	cough	PTB	on adm	No	187	No	No	Ceftriax, DOTS	10days	Survived	23/234/58	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
103	95208/10	31	F	No	Yes	No	Yes	No	No	Nil	Nil	4years	Yes	234	No	No	Ceftriax	3days	Survived	38/26/3	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
104	252706/12	29	M	Yes	No	No	Yes	No	No	Nil	Nil	3yrs	Yes	203	No	No	Ceftriax	3days	Survived	40/20/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
105	243306/12	36	M	Yes	No	No	Yes	No	No	Nil	Nil	3yrs	Yes	311	No	No	Ceftriax	3days	Survived	23/234/58	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
106	76507/11	28	F	No	No	Yes	Yes	No	No	Nil	oral thrush	3yrs	Yes	49	No	No	Amp-B	3days	Died	43/65/3	Neg	Few YLO	Neg	Growth (24 hrs)	Neg	white col	NA	NA	NA	NA	NA	NA	NA
107	267606/12	38	M	Yes	No	No	Yes	No	No	Nil	Nil	6months	No	296	No	No	Ceftriax	5days	Survived	36/20/-	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
108	100403/12	42	M	No	No	Yes	Yes	No	No	cough	PTB	4years	No	303	Yes	No	Ceftriax, DOTS	10days	Survived	40/20/4	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
109	85204/12	44	M	No	No	No	Yes	No	No	L hemiparesis, urine incontinence	oral thrush	Newly diag	No	34	No	No	Amp-B	10days	Survived	47/65/108	positive	pus cells+	positive	Growth (72 hrs)	Pos	brown col	NG	NG	0.25	0.064	1	4	0.06

110	316706/12	35	M	No	Yes	Yes	No	No	No	Nil	Nil	1yr	No	199	No	No	Ceftriax	5days	Survived	18/40/l	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
111	181206/12	28	M	Yes	Yes	No	No	No	No	Nil	Nil	4years	Yes	160	Yes	No	Ceftriax	5days	Survived	23/234/58	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
112	93306/12	35	M	No	Yes	No	Yes	No	No	Nil	oral thrush	4years	Yes	304	Yes	No	Ceftriax	5days	Survived	43/65/3	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
113	199905/12	33	F	Yes	No	No	Yes	No	No	Nil	oral thrush	4years	Yes	331	No	No	Ceftriax	5days	Survived	38/26/3	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
114	176506/12	45	M	Yes	Yes	No	No	No	No	Nil	Nil	2years	Yes	295	No	No	Ceftriax	3days	Survived	40/20/4	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
115	40904/11	27	M	No	No	Yes	Yes	No	No	Nil	Nil	2years	No	128	No	No	Ceftriax	3days	Survived	23/234/58	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
116	183906/12	38	M	No	No	Yes	Yes	No	No	Nil	Nil	2years	No	404	No	No	Ceftriax	3days	Survived	38/26/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
117	307306/11	28	M	Yes	Yes	No	No	No	No	Nil	Nil	2years	Yes	386	No	No	Ceftriax	3days	Survived	10/148/65	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
118	86101/12	26	F	No	No	Yes	No	No	No	Nil	Nil	2years	Yes	179	No	No	Ceftriax	5days	Survived	40/20/4	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
119	40704/12	25	M	No	Yes	No	No	No	No	Nil	Nil	2years	Yes	241	No	No	Ceftriax	5days	Survived	43/65/3	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
120	156407/12	45	M	Yes	Yes	No	No	No	No	cough	PTB	4years	Yes	32	Yes	No	Ceftriax, DOTS	10days	Died	38/26/3	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
121	176506/12	28	M	Yes	Yes	No	No	Yes	No	Nil	Nil	1yr	No	209	No	No	Ceftriax	5days	Survived	28/32/12	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
122	325503/12	40	M	No	No	Yes	No	No	No	Nil	Nil	on adm	No	29	No	No	Ceftriax	3days	Died	26/24/8	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
123	82609/12	35	M	No	No	Yes	No	No	No	Nil	Nil	2years	Yes	179	No	No	Ceftriax	5days	Survived	40/20/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
124	246207/12	37	M	Yes	Yes	No	No	No	No	Nil	Nil	4years	Yes	154	No	No	Ceftriax	3days	Survived	43/65/3	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
125	176506/12	40	M	No	No	Yes	No	No	No	Nil	Nil	2years	Yes	138	No	No	Ceftriax	5days	Survived	61/50/24	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
126	333907/12	39	M	Yes	Yes	Yes	No	No	No	Nil	Nil	4years	Yes	285	No	No	Ceftriax	5days	Survived	38/26/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
127	334987/12	42	M	No	Yes	Yes	No	No	No	Nil	Nil	1yr	No	314	No	No	Ceftriax	3days	Survived	38/26/3	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
128	335207/12	43	M	No	Yes	Yes	No	No	No	Nil	Nil	2years	Yes	308	No	No	Ceftriax	5days	Survived	40/20/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
129	145406/12	33	F	No	No	Yes	No	No	No	cough	PTB	4years	No	267	Yes	No	Ceftriax, DOTS	10days	Survived	26/24/8	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
130	220803/12	43	M	No	Yes	Yes	No	No	No	Nil	PTB	2years	Yes	not tested	Yes	No	Cat II DOTS, Amp-B	1day	Died	43/65/3	Neg	occ. pus cells, YLO	Positive	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
131	283611/07	35	M	No	No	Yes	Yes	No	No	cough	PTB	1yr	No	12	No	No	Ceftriax, DOTS	10days	Died	28/32/12	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
132	99891	25	F	No	No	Yes	Yes	No	No	Nil	nil	3 yrs	no	56	no	No	Amp-B	7days	Survived	44/60/22	Neg	Neg	Positive	Growth (5 days)	pos	brown col	NG	NG	0.12	0.016	2	8	0.06
133	145406/12	33	M	Yes	Yes	Yes	No	No	No	cough	PTB	2years	Yes	217	No	No	Ceftriax, DOTS	10days	Survived	38/26/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
134	41208/12	43	M	Yes	Yes	No	No	No	No	Nil	Nil	2years	Yes	187	No	No	Ceftriax	5days	Survived	61/50/24	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
135	79308/12	37	F	No	No	Yes	Yes	No	No	Nil	Nil	on adm	No	456	No	No	Ceftriax	3days	Survived	38/26/3	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
136	102908/12	38	M	Yes	Yes	Yes	No	No	No	Nil	Nil	1yr	No	237	No	No	Ceftriax	5days	Survived	28/32/12	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
137	114208/12	44	M	No	No	Yes	Yes	Yes	No	Nil	Nil	2years	Yes	296	No	No	Ceftriax	3days	Survived	40/20/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
138	224207/12	29	M	No	Yes	Yes	No	No	No	Nil	Nil	2years	Yes	302	No	No	Ceftriax	5days	Survived	38/26/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
139	151708/12	37	M	No	Yes	No	Yes	No	No	Nil	Nil	2years	Yes	169	No	No	Ceftriax	3days	Survived	28/32/12	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
140	322807/08	32	F	No	No	Yes	Yes	No	No	cough	PTB	2years	Yes	185	No	No	Ceftriax, DOTS	10days	Survived	26/24/8	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
141	1407/12	42	M	Yes	Yes	No	No	No	No	cough	PTB	6months	No	219	No	No	Ceftriax, DOTS	10days	Survived	40/20/4	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
142	121604/04	44	M	Yes	No	No	Yes	Yes	No	Nil	Nil	on adm	No	220	No	No	Ceftriax	5days	Survived	38/26/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA

143	194508/12	30	M	No	Yes	Yes	No	No	No	Nil	Nil	1yr	Yes	308	No	No	Ceftriax	3days	Survived	28/32/12	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
144	126108/04	36	M	No	Yes	Yes	No	No	No	Nil	Nil	1yr	Yes	367	No	No	Ceftriax	3days	Survived	10/148/65	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
145	514205/05	47	M	No	Yes	Yes	No	No	No	cough	PTB	2years	No	289	No	No	Ceftriax, DOTS	10days	Survived	61/50/24	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
146	226808/12	45	M	No	Yes	Yes	No	No	No	Nil	Nil	1yr	No	198	No	No	Ceftriax	5days	Survived	40/20/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
147	169604/12	42	M	Yes	Yes	No	No	No	No	Nil	oral thrush	3mon	No	115	No	No	Amp-B	10days	Survived	13/183/205	Neg	No pus cells	positive	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
148	129112/08	32	F	Yes	Yes	Yes	No	No	No	visual dist+	Nil	1yr	No	202	No	No	Ceftriax	5days	Survived	26/24/8	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
149	237608/12	28	M	Yes	Yes	No	No	No	No	Nil	Nil	2years	No	219	No	No	Ceftriax	3days	Survived	40/20/4	Neg	No pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
150	315608/12	24	F	No	No	Yes	No	No	No	Nil	Nil	2years	Yes	312	No	No	Ceftriax	3days	Survived	38/26/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
151	114208/12	44	M	No	No	No	Yes	No	No	Nil	Nil	2years	Yes	176	No	No	Ceftriax	5days	Survived	61/50/24	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
152	291301/05	42	M	Yes	Yes	Yes	No	No	No	Nil	Nil	2years	Yes	not tested	No	No	Ceftriax	3days	Died	40/20/4	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
153	68404/12	28	M	No	Yes	Yes	No	No	No	Nil	Nil	2years	Yes	160	No	No	Ceftriax	3days	Died	38/26/3	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
154	293611/11	38	F	No	Yes	No	Yes	No	No	visual dist+	Nil	2years	Yes	401	No	No	Ceftriax	5days	Survived	40/20/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
155	327507/12	22	M	No	Yes	Yes	Yes	Yes	No	Nil	oral thrush	on adm	No	25	No	No	Amp-B	2 days	Died	36/43/-	Positive	many pus cells, YLO	Positive	Growth (48 hrs)	Pos	Brown col	NG	NG	0.25	0.032	1	4	0.12
156	66709/10	40	M	Yes	Yes	No	No	No	No	Nil	Nil	2years	Yes	24	No	No	Ceftriax	3days	Survived	26/24/8	Neg	occ. pus cells	Positive	Growth +	NA	NA	NA	NA	NA	NA	NA	NA	NA
157	126108/04	36	M	Yes	No	Yes	No	No	No	Nil	Nil	6months	No	210	No	No	Ceftriax	5days	Survived	38/26/3	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
158	546207/06	46	M	No	Yes	Yes	No	No	No	Nil	Nil	1yr	No	145	No	No	Ceftriax	3days	Survived	61/50/24	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
159	362908/08	43	M	Yes	Yes	Yes	No	No	Yes	visual dist+	Nil	5years	Yes	89	Yes	Yes	Amp-B	7days	Survived	74/67/50	Neg	moderate pus cells	Positive	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
160	311403/07	55	M	No	Yes	No	Yes	No	No	Nil	Nil	1yr	No	139	No	No	Ceftriax	5days	Survived	40/20/4	Neg	occ. pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
161	32882	40	M	Yes	Yes	No	No	No	No	Nil	Nil	6months	No	297	No	No	Ceftriax	5days	Survived	44/18/8	Neg	No pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
162	72682	22	M	No	Yes	No	Yes	No	No	Nil	No	1yr	No	312	NA	No	Ceftriax	3days	Survived	46/20/6	Neg	No pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
163	84772	45	M	Yes	Yes	Yes	No	No	No	Thrush	DM	2yrs	Yes	289	NA	No	Ceftriax	3days	Survived	36/20/-	Neg	No pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
164	86985	39	M	No	Yes	No	Yes	Yes	Yes	Nil	diarrhoea	2 1/2yrs	Yes	31	Yes	Yes	Am-B	2days	Died	18/40/l	Pos	moderate pus cells	Positive	48hrs	NA	NA	NA	NA	NA	NA	NA	NA	NA
165	201007/12	41	M	No	Yes	No	No	No	No	paraparesis, blurring vision, fits (GTCS)	Nil	2 yrs	Yes	24	Yes	No	Amp-B	10 days	Survived	19/34/26	Positive	moderate pus cells	Positive	Growth (7 days)	Pos	Brown col	NG	NG	0.12	0.016	0.5	1	0.06
166	91235	17	M	Yes	No	Yes	No	No	No	Seizure	Nil	17yrs	Yes	194	No	No	Ceftriax	4days	Survived	50/24/4	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
167	98761	28	M	No	Yes	No	Yes	No	No	Nil		6months	No	485	No	No	Ceftriax	3days	Survived	44/23/2	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
168	109984	39	M	Yes	Yes	Yes	No	Yes	No	Nil	No	2yrs	No	376	No	No	Ceftriax	5days	Survived	18/30/l	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
169	92432/12	23	F	Yes	Yes	No	No	No	No	Nil	Nil	2years	Yes	36	No	No	Ceftriax	5days	Died	10/148/65	Neg	moderate pus cells	Neg	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
170	93107/11	41	M	Yes	Yes	No	No	No	No	swelling Left wrist	PTB	2yrs	Yes	62	no	No	Am-B	7days	Survived	61/50/24	Pos	Pus cells, YLO	Positive	Growth (72hrs)	Pos	Brown col	NG	NG	0.25	0.032	1	4	0.25
171	28939	40	M	No	Yes	Yes	Yes	Yes	No	Nil	Nil	2years	Yes	99	No	No	Ceftriax	3days	Survived	45/32/12	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
172	33955	55	M	Yes	Yes	No	No	No	No	FND+	Nil	1yr	No	301	No	No	Ceftriax	3days	Survived	26/24/8	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
173	37029	39	M	No	Yes	Yes	No	No	No	FND+	Nil	2yrs	Yes	299	No	No	Ceftriax	3days	Survived	38/26/3	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
174	37724	24	M	Yes	Yes	No	Yes	No	No	Nil	Nil	on adm	No	119	No	No	Ceftriax	2days	Died	24/24/2	Neg	occ. pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA
175	100202/12	27	F	No	No	Yes	Yes	No	No	Nil	Nil	2years	Yes	151	No	No	Ceftriax	5days	Survived	18/40/l	Neg	moderate pus cells	Negative	NG	NA	NA	NA	NA	NA	NA	NA	NA	NA